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Cell
Communication
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Cell Communication in Nervous and Immune System

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Series Editors

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Cell Communication in Nervous and Immune System

With 28 Figures, 8 in Color, and 3 Tables

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Preface

Signal exchange between cells is a key feature of life from humble monads to human beings. Appropriate communication is of particular importance between cells of multi-cellular organisms. Various basic mechanisms of cell-cell communication have evolved during phylogenesis, which were subject to organ, tissue and cell type-specific adaptation. These mechanisms range from long-distance communication via hormones to more and more local processes, e.g. via cytokines, chemokines or neuromodulators/neurotransmitters, and eventually direct physical interactions of molecules anchored at cell surfaces. Accordingly, highly specialized transient or stable cell-cell contact sites have evolved that mediate signaling between cells. With few exceptions (e.g. lipophilic hormones, gases) intercellular communication depends on specific signal detection devices at the cell surface coupled to a signal transduction apparatus that mediates the signal transfer across the cell membrane and activates intracellular effector systems, which generate intracellularly decipherable signals.

Prime examples for tissues of intensely communicating cells are the nervous and the immune systems. Although at the first glance these systems appear very different, both have developed sophisticated mechanisms for the formation of memory, though of quite different quality and significance for the organism. Memory formation in the immune system serves the recognition and tolerance of the organism's own cells and tissues as well as the effective recognition of and defense from invading pathogens. It is based on a complex network of cellular communication and signaling processes between cells of this "dispersed" organ and with target cells. Brain mechanisms of learning and memory, on the other hand, are indispensable for survival of an organism in its natural and social environment. They are based on the function and plasticity of the probably most complex cell junction: the chemical synapse. However, other cell-cell connections, such as gap junctions or specialized neuron-glia interaction sites, play an essential part in brain performance and plasticity.

This collection of reviews, contributed by internationally recognized immunologists and molecular and cellular neurobiologists, juxtaposes cellular communication devices and signaling mechanisms in the immune and the nervous system and discusses mechanisms of interaction between the two systems, the significance of which has only been fully appreciated in recent years.

Thus messengers produced by one of the two systems, such as cytokines or neuropeptides, can modulate cellular communication in the other system as well. Moreover, the central nervous system (CNS) has long been considered an immune-privileged organ lacking the classical immune response. Based on recent studies this view had to be revised and refined, and the particular role of the immune system in neuropathological as well as in neuroprotective and neurorepair processes has been recognized. This implies that the potentially harmful effects of the immune system in the CNS have to be tightly controlled by precise communication between cells of neural and immune systems.

The first four review articles deal with chemical synapses of the CNS. This highly sophisticated asymmetric cell–cell contact is designed for particular communication between neurons via chemical substances, the neurotransmitters. Neurotransmitters are stored in little membranous containers, i.e. synaptic vesicles, and released from the presynaptic cell in response to incoming electrical signals in a regulated manner. Different postsynaptic devices have evolved to detect excitatory (the first chapter) or inhibitory (the second chapter) transmitters and transduce the signals into the postsynaptic cell. Also the site of regulated neurotransmitter release from the presynaptic neuron—the active zone—is a complex molecular machine that organizes the synaptic vesicle cycle (the third chapter). The gap between the pre- and the postsynaptic membranes, the synaptic cleft, is a specialized extracellular compartment arranged by various cell adhesion molecules and components of the extracellular matrix that is thought to contribute importantly to synaptic assembly and plasticity (the fourth chapter).

Though known for more than 50 years, the electrical synapses have had a shadowy existence for a long time and only during recent years have their identity and their physiological relevance been studied in more detail. The fifth chapter discusses the role of gap junctions that form electrical synapses in the CNS. The next chapter discusses another intriguing cell–cell contact site that determines the capacity and efficacy of the vertebrate nervous system is the neuron-glia interaction at the so-called nodes of Ranvier, which facilitates rapid propagation of electrical signals along myelinated axons.

Also within the immune system the term “synapse” has been meanwhile well established. Here, the so-called immunological synapse describes the molecular and biophysical events that occur when immunocompetent cells interact with each other at the beginning of the adaptive immune response. T-cells, the major components of the adaptive immune system are by themselves incapable of detecting complete bacteria or viruses. Rather, the major structure on the T-cell surface that initiates T-cell activation, the T-cell receptor (TCR) only recognizes small, 9 to 12 amino acid-long bacterial or viral (antigenic) peptides. These have to be generated by particular immunocompetent cells, which have collectively been termed antigen presenting cells (APCs). Although it is well known that B-cells, dendritic cells and macrophages rep-

resent the major types of APCs that activate T-cells, it is still unclear whether, for example, endothelial cells, which are spread throughout the whole body, are also capable of presenting antigens to T-cells, at least in particular organs such as the liver, or under particular conditions such as inflammation. These questions are addressed in the seventh chapter, which also discusses the immunological consequences of the interaction between T-cells and endothelial cells.

Importantly, the mere generation of antigenic peptides is not sufficient to activate T-cells. This is due to the fact that the TCR only signals when antigenic peptides are presented to the T-cell by APCs in conjunction with self-molecules, the so-called major histocompatibility complex (MHC) molecules. The detection of antigen/MHC by the TCR occurs at the beginning of the immune response at the interface between the T-cell and the APC and this first physical contact between the two cells induces the formation of the immunological synapse. Consequently, the immunological synapse is a highly dynamic structure that changes its morphology and molecular composition during the initial phase of the immune response. During the past decade numerous groups have begun to dissect the molecular events that either regulate the formation of the immunological synapse or occur after immunological synapse formation by applying sophisticated microscopic and biochemical techniques. As a result, several models of the molecular composition and the function of the immunological synapse have evolved. The eighth and ninth chapters focus on the biophysics and the morphological changes of the immunological synapse under different conditions of stimulation and further discuss the role of the immunological synapse during T-cell activation. While these two articles primarily deal with the dynamics of APC/T-cell interactions and the morphological changes of the immunological synapse on the microscopic level, the following two chapters focus on the signaling events that regulate particular aspects of immunological synapse formation and T-cell activation. The first of these discusses alterations of the cytoskeleton and the second the regulation of intimate membrane contacts via adhesion molecules and integrins.

The final two chapters shed some light on the communication between the immune and the nervous system and the control of immune responses in the nervous system. To gain access to the CNS, immune cells have to cross the blood-brain barrier provided by composed of endothelial cells. How this process is mediated and controlled under physiological and pathological conditions is discussed in the penultimate chapter. Endocannabinoids, the endogenous ligands for the “Marihuana” receptors, seem to be intricately involved in the neural control of the immune system. The current view of how the CNS endocannabinoid system contributes to the immune surveillance is discussed in the final chapter.

This book is dedicated to our colleague Werner Hoch, who intended to contribute an article on the neuromuscular junction, the supposedly best-

studied vertebrate synapse and long-time focus of Werner's scientific work. Werner passed away last summer—unexpectedly and much too early for all of us.

Magdeburg, 2006

Eckart D. Gundelfinger
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Molecular Organization and Assembly of the Postsynaptic Density of Excitatory Brain Synapses

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Abstract The postsynaptic density (PSD) is a postsynaptic membrane specialization at excitatory synapses. The PSD is made of macromolecular multiprotein complexes, which contain a variety of synaptic proteins including membrane, scaffolding, and signaling proteins. By coaggregating with postsynaptic cell adhesion molecules, PSD proteins promote the formation and maturation of excitatory synapses. PSD proteins organize signaling pathways to coordinate structural and functional changes in synapses, and they regulate trafficking and recycling of glutamate receptors, which determines synaptic strength and plasticity. Synaptic activity dynamically regulates the assembly of the PSD through mechanisms including protein phosphorylation, palmitoylation, and protein degradation. PSD proteins associate with diverse motor proteins, suggesting that they function as adaptors linking motors to their specific cargoes.

1

Introduction

The postsynaptic density (PSD) is an electron-dense postsynaptic membrane specialization located in dendritic spines, where most of the excitatory synaptic transmission occurs. During the last decade, a large number of PSD components have been identified. In this review, we will not give a comprehensive description of known PSD proteins and synaptic mechanisms; rather, we will focus on how key PSD proteins are assembled to form the PSD and coordinate structural and functional plasticity of excitatory synapses.

2

Components of the PSD

PSD proteins are characterized by their biochemical enrichment in PSD fractions and by the presence of multiple protein–protein interaction domains (Fig. 1). These proteins were mostly identified through molecular and cellular methods, such as yeast two-hybrid screening. However, recent proteomic studies have identified a large number of PSD proteins, which include both

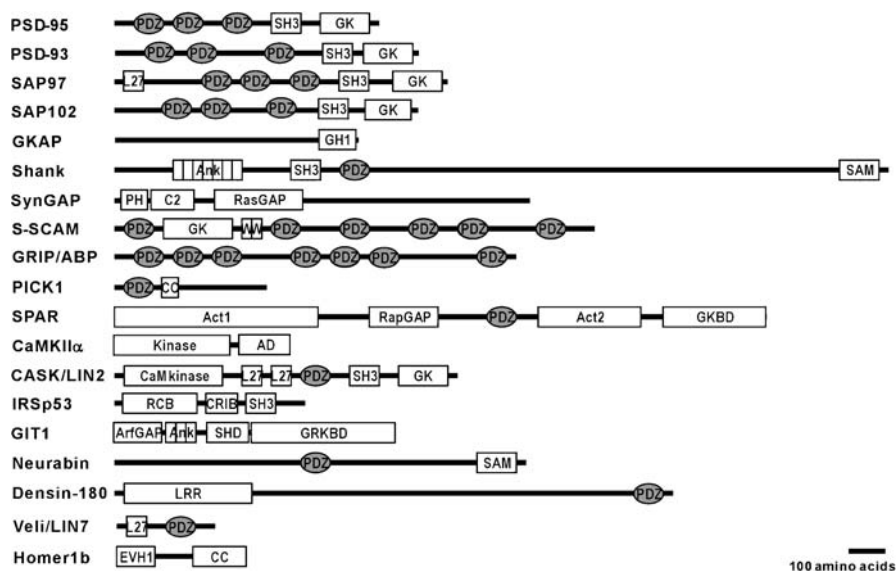


Fig. 1 Schematic diagram of PSD proteins. Examples of PSD proteins are shown along with their domain structures. PDZ domains are shown as gray ellipses. Other domains are indicated: Act1, actin regulatory domain 1; Act2, actin regulatory domain 2; AD, association domain; Ank, ankyrin repeats; ArfGAP, Arf GTPase-activating protein; C2, calcium/lipid binding domain 2; CAM kinase, Ca^{2+} /calmodulin-dependent kinase (CAME)-like domain; CC, coiled coil domain; CRIB, Cdc42/Rac-interactive binding; EVH1, ENA/VASP homology domain 1; GH1, GKAP homology domain 1; GK, guanylate kinase-like domain; GKBD, PSD-95 GK binding domain; GRKBD, GRK2 binding domain; Kinase, serine/threonine kinase domain; L27, domain initially found in LIN2 and LIN7; PDZ, PSD-95/Dlg/ZO-1 domain; LRR, leucine rich repeat; PH, pleckstrin homology domain; RapGAP, Rap GTPase-activating protein; RasGAP, Ras GTPase-activating protein; RCB, Rac binding domain; SAM, sterile α motif; SH3, Src homology 3 domain; SHD, Spa2 homology domain; WW, domain with two conserved Trp (W) residues. Proteins: CaMKII α , Ca^{2+} /calmodulin-dependent kinase II alpha; CASK/LIN2, vertebrate homolog of lin2; GIT1, GRK-interacting protein 1; GKAP, GK associated protein; GRIP/ABP, glutamate receptor interacting protein/AMPA receptor binding protein; IRSp53, insulin receptor tyrosine kinase substrate p53; PICK1, protein interacting with C-kinase; PSD-93, postsynaptic density protein 93; PSD-95, postsynaptic density protein 95; SAP97; synapse-associated protein 97, SAP102, synapse-associated protein 102; Shank, SH3 and ankyrin repeat-containing protein; SPAR, spine-associated RapGAP; S-SCAM, synaptic scaffolding molecule; Veli/LIN7, vertebrate homolog of lin7. Scale bar, 100 amino acids

known and novel proteins (Husi et al. 2000; Walikonis et al. 2000; Satoh et al. 2002; Jordan et al. 2004; Li et al. 2004; Peng et al. 2004; Yoshimura et al. 2004; Collins et al. 2005). Although it is not yet clear whether the newly identified proteins represent real PSD components, the proteomic results have certainly enhanced our understanding of the molecular profile of the PSD. For instance, a recent proteomic study identified a total of 374 proteins as components

of the PSD. These proteins were further divided into distinct groups based upon their known or predicted functions (Peng et al. 2004), which include receptors/channels, cell adhesion molecules (CAMs), scaffolding proteins, actin cytoskeleton proteins, membrane trafficking proteins, GTPases and regulators, protein kinases/phosphatases, translation proteins, and mitochondrial proteins.

Proteomic analysis has provided additional information on PSD proteins such as the patterns of their phosphorylation and their relative abundance. For instance, a recent quantitative mass spectrometry study revealed that PSD-95 is approximately five-fold more abundant in the PSD than the NR1 subunit of NMDA receptors (NMDARs) (Peng et al. 2004). The greater abundance of PSD-95 family proteins may explain why neurons from PSD-95-deficient mice show normal synaptic NMDAR localization (Migaud et al. 1998) and why PSD-95 overexpression in cultured neurons does not enhance synaptic localization of NMDARs (El-Husseini et al. 2000a).

3

Assembly of the PSD

Abundant multidomain PSD proteins are thought to play a key role in PSD assembly. The PSD-95 family is one of the most extensively studied group of PSD proteins and includes PSD-95/SAP90, SAP97, PSD-93/chapsyn-110, and SAP102. These proteins have several domains for protein-protein interactions, including the PDZ domain, an approximately 90-aa module for binding to C-terminal peptides; the SH3 domain; and the guanylate kinase (GK) domain (Fig. 1). Accordingly, PSD-95 associates with diverse membrane and signaling proteins at excitatory synapses (Funke et al. 2004; Kim and Sheng 2004). In addition, PSD-95 binds to other scaffolding proteins such as GKAP/SAPAP and Shank/ProSAP, which are localized in the deeper layers of the PSD. These are linked to additional synaptic proteins, therefore adding to the size of the PSD complexes.

Assembly of the PSD may involve the sequential recruitment of PSD proteins. Negative-stain electron microscopy reveals that PSD-95 has a C-shaped conformation, in which the *N*- and *C*-terminal regions bind to each other (Nakagawa et al. 2004). Consistently, the weak binding of full-length PSD-93 to MAP1A, which strongly binds to the isolated GK domain of PSD-93, is partially restored by the binding of peptides to the *N*-terminal PDZ domains (Brenman et al. 1998), suggesting that binding of an initial ligand molecule to PSD-95 changes it from a closed to an open conformation, promoting the binding of a second ligand molecule. This notion is supported by the finding that the binding of GKAP changes the conformation of Shank (Romorini et al. 2004).

Protein multimerization may contribute to the organization of the PSD. For example, homo- and/or hetero-multimerization has been observed for several

PSD proteins, including PSD-95, SAP97, Shank, Homer, GRIP/ABP, PICK1, and GIT1 (Funke et al. 2004; Kim and Sheng 2004; Romorini et al. 2004). Multimerization of PSD proteins may increase the number and diversity of docking sites in the PSD and the affinity and stability of the protein–protein interactions.

PSD assembly seems to involve a gradual recruitment of the components (Bresler et al. 2001, 2004; Marrs et al. 2001; Okabe et al. 2001a,b; Bresler et al. 2004), in contrast to the generation of presynaptic active zones by recruitment of a small number of preassembled mobile packets (Ziv and Garner 2004). For instance, live imaging of GFP-labeled PSD proteins including NR1, PSD-95, Shank2/ProSAP1, and Shank3/ProSAP2 suggests that they are gradually recruited (Bresler et al. 2001; Bresler et al. 2004), although modular transport of NR1 and PSD-95 has been reported (Prange and Murphy 2001; Washbourne et al. 2002; Washbourne et al. 2004a). This gradual accumulation may contribute to the homeostatic changes at excitatory synapses.

4

Synaptic Adhesion and PSD Proteins

The assembly of the PSD coincides with the formation of presynaptic structures (Friedman et al. 2000; Marrs et al. 2001; Okabe et al. 2001a), indicative of a trans-synaptic interaction. Neuroligin is a postsynaptic CAM that promotes synapse formation by interacting with presynaptic β -neurexin through its ectodomain and with PSD-95 through its PDZ-binding C-terminus (Washbourne et al. 2004b). The initial clustering of neuroligin induces co-aggregation of PSD-95 and other postsynaptic proteins (Graf et al. 2004; Nam and Chen 2005). Thus, CAM-interacting PSD proteins seem to couple extracellular adhesion signals to the development of postsynaptic multiprotein complexes.

PDZ interactions may be generally involved in CAM-mediated synapse formation. SynCAM associates with synaptic PDZ proteins including CASK and syntenin (Biederer et al. 2002). Dasm1, a synaptic Ig superfamily member involved in the regulation of dendritic arborization and synaptic maturation, binds to Shank and S-SCAM PDZ proteins (Shi et al. 2004a, b). Sidekick, a synaptic CAM that determines synaptic connectivity in the retina, has a PDZ domain-binding motif at its C-terminus (Yamagata et al. 2002).

In addition to being passively recruited to sites of CAM aggregation, PSD proteins seem to have some active roles. Neuroligin isoforms show distinct synaptic localization: neuroligin-1 is located mainly at excitatory synapses whereas neuroligin-2 is located at inhibitory synapses (Song et al. 1999; Graf et al. 2004; Varoqueaux et al. 2004; Chih et al. 2005; Levinson et al. 2005). Consistently, neuroligins regulate the development of both excitatory and inhibitory synapses (Graf et al. 2004; Prange et al. 2004; Chih et al.

2005; Levinson et al. 2005). Of note, overexpression of PSD-95 translocates neuroligin-2 to excitatory synapses (Graf et al. 2004; Levinson et al. 2005) and increases the ratio of excitatory to inhibitory synapses (Prange et al. 2004). Conversely, siRNA knockdown of PSD-95 decreases the ratio of excitatory to inhibitory synapses (Prange et al. 2004). However, synaptic localization of neuroligin-1 does not require its PSD-95-binding C-terminus (Dresbach et al. 2004), suggesting that PSD-95 may promote synaptic localization of neuroligins through PDZ-independent mechanisms. Although further details remain to be determined, these results suggest that PSD proteins may actively drive synaptic CAMs to excitatory synapses and determine the balance between excitatory and inhibitory synapses.

5

Membrane Proteins and PSD Proteins

PSD proteins have diverse effects on interacting membrane proteins. PSD-95 clusters NMDARs, stargazin (a transmembrane AMPA receptor [AMPA] regulatory protein or TARP), and potassium channels in heterologous cells (Chen et al. 2000; Kim and Sheng 2004). PSD-95 promotes NMDAR insertion to the surface membrane, decreases channel internalization, protects NMDARs from calpain-mediated cleavage, and decreases glutamate sensitivity and desensitization of NMDARs (Yamada et al. 1999; Li et al. 2003; Dong et al. 2004; Lin et al. 2004). In addition, PSD-95 promotes synaptic localization of the NR2A subunit of NMDARs but reduces that of NR2B (Losi et al. 2003), changing the subunit composition of synaptic NMDARs. Stargazin mutants lacking PSD-95 binding lose their synaptic localization (Chen et al. 2000; Chetkovich et al. 2002). A mutant GluR2 AMPAR subunit that lacks GRIP binding shows a reduced synaptic localization (Osten et al. 2000). At the single channel level, PSD-95 increases the open probability of NMDAR (Lin et al. 2004) and decreases the conductance of Kir2.3 K⁺ channel (Nehring et al. 2000). Together, these results suggest that PSD proteins regulate the synaptic delivery, clustering, stabilization, subunit composition, ligand sensitivity, endocytosis, and intrinsic functional properties of interacting membrane proteins.

6

Spine Formation and PSD Proteins

Polymerization of F-actin in dendritic spines regulates spine morphogenesis and synaptic plasticity (Segal 2005). The Rac1, RhoA, and Cdc42 Rho family small GTPases are important regulators of F-actin remodeling in spines. For instance, Rac1 promotes spine density and maturation, whereas RhoA

has an opposite role (Luo et al. 1996; Nakayama et al. 2000; Tashiro et al. 2000; Tashiro and Yuste 2004).

PSD proteins directly associate with the Rac1 signaling pathway proteins (Fig. 2), promoting their recruitment to synapses and possibly facilitating their functional coupling. GRIP1 interacts with the EphB receptor tyrosine kinase (Torres et al. 1998), which regulates spine morphogenesis through its action on downstream effectors including the Rac1 guanine-nucleotide exchange factor (GEF) kalirin-7 and the Cdc42 GEF intersectin (Irie and Yamaguchi 2002; Henkemeyer et al. 2003; Penzes et al. 2003). NMDAR associates with ligand-activated EphB receptor (Dalva et al. 2000) as well as with the Rac1 GEF Tiam1 (Tolias et al. 2005). PSD-95 interaction is required for synaptic localization of kalirin-7 (Penzes et al. 2001). Shank promotes synaptic localization of β PIX, a GEF for Rac1/Cdc42, and β PIX-associated PAK1 (p21-activated kinase) (Park et al. 2003), a downstream effector of Rac1 and Cdc42 acting on LIMK-1 and MLC for actin regulation (Meng et al. 2002; Zhang et al. 2005). GIT1, a multi-domain protein enriched in the PSD and implicated in AMPAR trafficking (Ko et al. 2003), also regulates synaptic localization of β PIX and spine morphogenesis (Zhang et al. 2003, 2005). Finally, both PSD-95

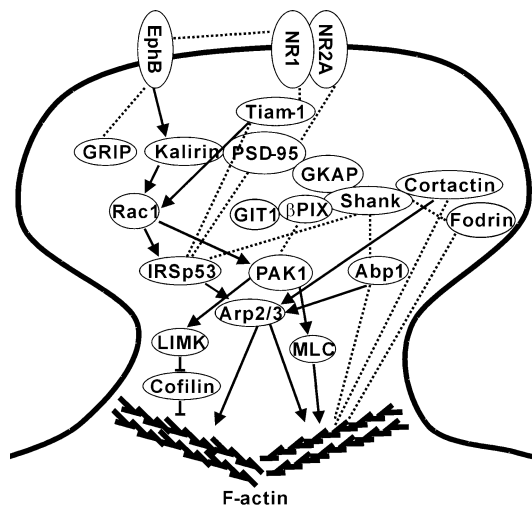


Fig. 2 Association of PSD proteins with Rac1 signaling pathway proteins. PSD proteins associate with Rac1 signaling pathway proteins and regulate their synaptic localization and/or activity, which may contribute to the organization of the Rac1 signaling pathway in dendritic spines. Specific protein–protein interactions are indicated by the direct contacts of proteins or *dotted lines*. Signaling flows are indicated by *arrows*. Abp1, actin-binding protein 1; Arp2/3, actin-related 2/3 complex; β PIX, PAK-interactive exchange factor; Cofilin, actin depolymerizing factor; EphB, ephrin receptor type B; LIMK, LIM kinase; MLC, myosin regulatory light chain; NR1, NMDA receptor subunit 1; NR2A, NMDA receptor subunit 2A; PAK1, p21-activated kinase

and Shank regulate the synaptic localization of IRSp53, a downstream effector of Rac1 and Cdc42 that regulates spine formation (Bockmann et al. 2002; Soltau et al. 2002; Choi et al. 2005).

In addition to Rac1 pathway proteins, PSD proteins associate with diverse actin remodeling or spine regulatory proteins. Shank interacts with the actin-binding protein Abp1 (Qualmann et al. 2004), the actin-crosslinking protein α -fodrin (Bockers et al. 2001), and cortactin (a protein promoting actin nucleation through Arp2/3) (Du et al. 1998). CASK binds to syndecan, a cell surface heparan sulfate proteoglycan, which requires its C-terminal PDZ-binding motif for spine promotion (Hsueh et al. 1998; Ethell and Yamaguchi 1999). Actin-binding proteins neurabin and spinophilin interact with Lfc, a Rho GEF that regulates spine morphology (Ryan et al. 2005).

Consistent with the implication of core PSD proteins in spine morphogenesis, overexpression of PSD-95 promotes spine density (El-Husseini et al. 2000a), whereas degradation of PSD-95 induced by the SNK polo-like kinase reduces spine numbers (Pak and Sheng 2003). Similarly, overexpression of Shank1 promotes maturation of spines (Sala et al. 2001), whereas siRNA knockdown of Shank3 reduces spine number (Roussignol et al. 2005).

In addition to promoting synaptic localization of actin remodeling proteins, PSD proteins regulate spine morphogenesis by functionally modulating their activity in spines. Synaptic localization of cortactin, SPAR (a GTPase-activating protein [GAP] for Rap), and SynGAP (Ras/Rap GAP) does not depend on their interaction with PSD-95 or Shank but rather on F-actin binding (Pak et al. 2001; Hering and Sheng 2003; Vazquez et al. 2004). Consistently, SynGAP requires PSD-95 interaction for its activity (Vazquez et al. 2004).

7

Postsynaptic Signaling and PSD Proteins

The strength and plasticity of excitatory synapses are regulated by signaling pathways in dendritic spines (Kennedy et al. 2005). The molecular organization of these pathways is coordinated by PSD proteins. A well-known example is the association of PSD-95 with neuronal nitric oxide synthase, which couples NMDAR activation to nitric oxide generation (Aarts et al. 2002; Bredt 2005).

SynGAP is a neuron-specific GAP for Ras and Rap small GTPases (Chen et al. 1998; Kim et al. 1998), which regulate AMPAR trafficking and synaptic plasticity (Zhu et al. 2002, 2005). SynGAP is activated by Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII)-dependent phosphorylation (Oh et al. 2004) and thus may couple NMDAR activation to the regulation of the Ras-ERK pathway and AMPAR trafficking.

Genetic analyses of mice have shed light on the function of SynGAP. Homozygote SynGAP-deficient mice die within a few days of birth (Komiya et al. 2002; Kim et al. 2003; Vazquez et al. 2004), and conditional SynGAP

knockout mice with a delayed loss of SynGAP show enhanced neuronal apoptosis (Knuesel et al. 2005). Also, heterozygotic mice exhibit increased basal ERK activity, increased synaptic AMPAR clustering, reduced long-term potentiation (LTP), and impaired spatial learning (Komiyama et al. 2002; Kim et al. 2003). Cultured neurons from homozygotic mice show an accelerated development of dendritic spines and synapses as well as an increase in spine size (Vazquez et al. 2004). These results suggest that SynGAP is a key regulator of neuronal development, synaptic structure and function, and memory.

How does SynGAP regulate synaptic function? A recent study demonstrates that SynGAP and CaMKII preferentially associate with NR2B but not NR2A (Kim et al. 2005), suggesting that SynGAP may contribute to the selective association of NR2B with long-term depression (LTD) (Liu et al. 2004). Consistently, SynGAP overexpression reduces AMPAR insertion into the plasma membrane, whereas siRNA knockdown of SynGAP prolongs NMDAR-mediated Ras-ERK activation. These results suggest that SynGAP couples the activation of NR2B-containing NMDARs to the inhibition of the Ras-ERK pathway and AMPAR surface insertion. Similarly, MUPP1, a protein with multiple PDZ domains, interacts both SynGAP and CaMKII (Krapivinsky et al. 2004), bringing the NMDAR-CaMKII complex into the vicinity of SynGAP. Disruption of the MUPP1-SynGAP interaction results in dephosphorylation of SynGAP and inactivation of p38 MAPK, suggesting that MUPP1 couples NMDAR activation to the regulation of SynGAP and the p38 MAPK signaling pathway.

AKAP79/150 is a synaptic scaffold that anchors cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and protein phosphatase 2B (calcineurin). Although AKAP79 binds to PSD-95 and SAP97 (Colledge et al. 2000), synaptic localization of AKAP79 depends on the binding of F-actin but not PSD-95 (Gomez et al. 2002). Thus, PSD-95 and/or SAP97 may bring AKAP-associated kinases and phosphatases close to their synaptic substrates, including the SAP97-bound GluR1 AMPAR subunit (Leonard et al. 1998). In support of this, the SAP97-AKAP79 complex promotes the basal phosphorylation of GluR1 on Ser 845 (Colledge et al. 2000), a key determinant of the function and recycling of AMPARs and synaptic plasticity (Banke et al. 2000; Ehlers 2000; Lee et al. 2000, 2003a). This complex also confers calcium- and protein phosphatase 2B-mediated downregulation of GluR1 currents (Tavalin et al. 2002). Consistently, disruption of the interaction between AKAP79/150 and cAMP-dependent protein kinase reduces synaptic AMPAR levels and occludes LTD (Snyder et al. 2005).

Tyrosine phosphorylation regulates NMDAR activity (Salter and Kalia 2004), AMPAR trafficking (Ahmadian et al. 2004; Hayashi and Haganir 2004), and synaptic localization of β -catenin, a protein that links cadherins to the actin cytoskeleton for the regulation of synaptic structure and function (Murase et al. 2002). PSD-95 associates with Src family non-receptor tyrosine kinases (Kalia and Salter 2003) and their upstream activator CAK β /Pyk2

(Huang et al. 2001), suggesting that PSD-95 recruits these kinases to their synaptic substrates. LAR, a synaptic receptor protein tyrosine phosphatase, binds to the multidomain protein liprin- α , which in turn associates with the AMPAR-GRIP complex (Wyszynski et al. 2002; Dunah et al. 2005). The phosphatase activity of LAR and the LAR-liprin- α -GRIP interaction are required for the maintenance of dendritic spines and excitatory synapses (Dunah et al. 2005). In addition, LAR directly dephosphorylates β -catenin and regulates its synaptic localization. These results suggest that liprin- α may link LAR to its specific substrates through protein-protein interactions.

8

Regulation of Synaptic Transmission and Plasticity by PSD Proteins

The role of PSD-95 family proteins in the regulation of synaptic transmission and plasticity has been studied extensively. Overexpression of PSD-95 enhances AMPAR-mediated excitatory postsynaptic currents (EPSCs) (El-Husseini et al. 2000a; Beique and Andrade 2003; Stein et al. 2003; Ehrlich and Malinow 2004). Conversely, siRNA knockdown of PSD-95 reduces AMPAR EPSCs (Nakagawa et al. 2004). Indicative of a role in LTP expression, PSD-95 overexpression drives GluR1 AMPAR to synapses, converts silent synapses into mature ones, occludes LTP, and enhances LTD (Beique and Andrade 2003; Stein et al. 2003; Ehrlich and Malinow 2004). PSD-95-deficient mice, however, exhibit an enhanced LTP and a reduced LTD, a finding that remains to be explained (Migaud et al. 1998; Yao et al. 2004). Because PSD-95 does not directly associate with AMPARs, the LTP-promoting effect of PSD-95 could be mediated by stargazin, which directly interacts with AMPARs and PSD-95 and regulates AMPAR trafficking and bidirectional synaptic plasticity (Chen et al. 2000; Schnell et al. 2002; Tomita et al. 2005a,b).

SAP97 promotes AMPA EPSCs and occludes LTP (Rumbaugh et al. 2003; Nakagawa et al. 2004), consistent with the implication that SAP97 participates in the trafficking of GluR1 AMPARs (Leonard et al. 1998; Hayashi et al. 2000; Sans et al. 2001). Conversely, siRNA knockdown of SAP97 depletes surface GluR1 and GluR2 and reduces EPSCs for AMPA and, notably, NMDA (Nakagawa et al. 2004), suggesting that SAP97 has a broader role in the regulation of synaptic function.

GRIP/ABP, PICK1, and NSF bind to the C-terminal region of AMPAR subunits and regulate their trafficking and recycling. Here we describe relatively recent findings, because previous results have been summarized in several excellent reviews (Barry and Ziff 2002; Malinow and Malenka 2002; Brecht and Nicoll 2003; Sheng and Hyoungh Lee 2003; Collingridge et al. 2004; Palmer et al. 2005).

GRIP/ABP family proteins, which include GRIP1 and GRIP2, contain multiple PDZ domains and are thought to help anchor GluR2/3 at synaptic or

intracellular sites (Daw et al. 2000; Osten et al. 2000). As with PSD-95, a splice variant of GRIP/ABP is directed to the plasma membrane and dendritic spines by palmitoylation in the *N*-terminus (DeSouza et al. 2002). Also, a region in the middle of GRIP/ABP including PDZ4-6 mediates association with intracellular membrane clusters (Fu et al. 2003).

GRIP/ABP interacts not only with AMPARs but also with diverse synaptic proteins. The association of GRIP with liprin- α and liprin- α -associated GIT1 is important for dendritic and surface clustering of AMPARs (Wyszynski et al. 2002; Ko et al. 2003). GRIP associates with GRASP-1, a neuronal Ras GEF (Ye et al. 2000), possibly coupling Ras signaling to AMPAR trafficking. GRIP interacts with Eph receptors and their ephrin ligands, regulating LTP (Contractor et al. 2002). The *in vivo* function of GRIP at synapses, however, has not yet been explored, partly because of the embryonic lethality of a GRIP1 knockout (Bladt et al. 2002; Takamiya et al. 2004).

Another PSD protein PICK1 binds to GluR2/3 and PKC α through its PDZ domain. PICK1 targets activated PKC α to spines (Perez et al. 2001), and phosphorylation of synaptic GluR2 on Ser 880 by PKC α decreases its binding to GRIP but not to PICK1 (Chung et al. 2000; Matsuda et al. 2000) and may lead to PICK1-dependent internalization of AMPARs (Perez et al. 2001) and LTD expression. Consistent with these findings, LTD induction causes the phosphorylation of GluR2 on Ser 880 (Matsuda et al. 2000; Kim et al. 2001), and synthetic peptides blocking the interaction of PICK1 with GluR2/3 inhibit the expression of LTD both in hippocampus and cerebellum (Xia et al. 2000; Kim et al. 2001). In addition to AMPARs, PICK1 and GRIP interact with kainate receptors (GluR5, GluR6 and GluR7), which are required for the maintenance of kainate receptor-mediated synaptic transmission (Hirbec et al. 2003). PICK1 also binds and regulates the trafficking of other membrane proteins including metabotropic glutamate receptors (Boudin et al. 2000; Dev et al. 2000; Hirbec et al. 2002; Perroy et al. 2002), UNC5H (netrin receptor) (Williams et al. 2003), dopamine plasma membrane transporter (Torres et al. 2001), and ErbB2 receptor tyrosine kinase (Jaulin-Bastard et al. 2001).

NSF, an ATPase involved in membrane fusion, binds to a C-terminal region of GluR2 that is distinct from the PICK1/GRIP-binding site. Inhibition of NSF binding to GluR2 using synthetic peptides causes a decrease in the surface expression of AMPARs, rundown of AMPA EPSCs, and occlusion of LTD (Palmer et al. 2005). NSF, with the aid of SNAP, disassembles the GluR2-PICK1 complex (Hanley et al. 2002), suggesting that NSF maintains synaptic AMPARs by inhibiting the PICK1-dependent removal of AMPARs. In addition, NSF has been shown to be involved in the recycling of internalized AMPARs, inhibiting their lysosomal degradation (Lee et al. 2004). AP2, a clathrin adaptor complex that mediates endocytosis, interacts with a C-terminal region of GluR2 that overlaps with the NSF-binding site and is required for NMDA-induced AMPAR internalization and hippocampal LTD (Lee et al. 2002), suggesting that AP2 is involved in regulated internalization of AM-

PARs. Of note, NMDAR activation promotes S-nitrosylation of NSF, binding of NSF to GluR2, and surface expression of GluR2, suggesting that this mechanism may contribute to NMDAR-dependent LTP induction (Huang et al. 2005). Recent reports have also shown that the interaction of NSF and PICK1 with GluR2 is required for activity-dependent delivery of GluR2-containing AMPARs into synapses in cerebellar stellate cells, contributing to the plastic changes in calcium permeability of AMPARs in these cells (Gardner et al. 2005; Liu and Cull-Candy 2005).

9

Dynamic Regulation of the Assembly of the PSD

A large number of PSD proteins contain the PDZ domain (Funke et al. 2004; Kim and Sheng 2004). Therefore, phosphorylation-dependent inhibition of PDZ interaction would be an efficient way of regulating PSD assembly. Indeed, C-terminal phosphorylation of potassium channels (Tanemoto et al. 2002), β 1-adrenergic receptor (Hu et al. 2002), and stargazin (Chetkovich et al. 2002; Choi et al. 2002) inhibits their PDZ-mediated interaction with PSD-95. The C-terminal phosphorylation of NR2B on Ser 1480 by casein kinase II disrupts interaction with PSD-95 and decreases the surface expression of NR2B (Chung et al. 2004). Similarly, PKC-dependent phosphorylation of GluR2 on Ser 880 disrupts its PDZ interaction with GRIP but does not affect its binding to PICK1 (Chung et al. 2000; Matsuda et al. 2000). Also, CaMKII phosphorylation of SAP97 on Ser 232 in the PDZ1 domain disrupts its interaction with NR2A (Gardoni et al. 2003), a process that may regulate synaptic localization of NMDARs.

PDZ-independent phosphorylation also regulates assembly of the PSD. Cdk5 phosphorylates PSD-95 in its *N*-terminal domain, inhibiting the multimerization and channel clustering activity of PSD-95 and reducing the size of synaptic PSD-95 clusters (Morabito et al. 2004). Also, CaMKII phosphorylates SAP97 on Ser 39 in the *N*-terminal L27 domain, promoting synaptic localization of SAP97 and GluR1 (Mauceri et al. 2004).

Palmitoylation occurs on diverse neuronal proteins, including receptors, scaffolding proteins, and signaling proteins (El-Husseini Ael and Bredt 2002). Palmitoylation of PSD-95 at the *N*-terminal residues Cys3 and Cys5 promotes synaptic localization of PSD-95, whereas synaptic activity leads to depalmitoylation and dispersal of PSD-95 (El-Husseini Ael et al. 2002). Chapsyn-110/PSD-93 and GRIP/ABP are similarly palmitoylated in their *N*-terminal regions (El-Husseini et al. 2000b; DeSouza et al. 2002). Recent studies have identified palmitoyl transferases that specifically modify PSD-95 and other synaptic proteins (Fukata et al. 2004; Huang et al. 2004).

Protein degradation by the ubiquitin-proteasome pathway seems to regulate the composition of the PSD. Mdm2, an E3 ubiquitin ligase, interacts with

and ubiquitinates PSD-95 (Colledge et al. 2003). The polo-like kinase SNK, which is induced by synaptic activity, phosphorylates the Rap GAP SPAR and induces loss of SPAR, PSD-95, and dendritic spines (Pak and Sheng 2003). Furthermore, it has been proposed that synaptic activity regulates the ubiquitination of PSD-95 and other scaffolding proteins, including GKAP, Shank, and AKAP79 (Ehlers 2003).

10

Transport of PSD Proteins by Motor Proteins

Most of the PSD proteins are thought to be translated in the cell body and then transported to their target synapses. The kinesin superfamily of microtubule-based motor proteins mediates molecular transport in neurons (Hirokawa and Takemura 2005). Although not as extensively studied as axonal transport, a variety of findings support the presence of dendritic transport of postsynaptic cargoes. In addition to the association of post-synaptic proteins with motor proteins (see below), the minus end-directed kinesin KIFC2 is detected mainly in dendrites (Hanlon et al. 1997; Saito et al. 1997). In fact, the movement of KIF1A and KIF17 kinesins has been directly visualized in the dendrites of living neurons (Guillaud et al. 2003; Lee et al. 2003b).

Because the known number of kinesins in the human genome (approximately 45 kinesins) is far less than that of their cargoes, multidomain adaptors are thought to function as “motor receptors”, linking kinesins to diverse cargoes through protein–protein interactions (Hirokawa and Takemura 2005). In neurons, synaptic scaffolding proteins seem to function as motor receptors during their delivery to synapses. In support of this, PSD-95, SAP97, and S-SCAM associate with the kinesin motor protein KIF1B α through a PDZ interaction (Mok et al. 2002). Distinct domains of SAP97 interact with the kinesin-like motor GAKIN and the actin-based motor myosin-VI (Wu et al. 2002; Asaba et al. 2003), which is implicated in the regulation of synapse formation and AMPAR trafficking (Osterweil et al. 2005). In addition, GKAP binds to myosin-V, linking myosin-V to GKAP-associated proteins including PSD-95 and Shank (Naisbitt et al. 2000). Furthermore, the LIN-10 PDZ protein links the kinesin motor KIF17 to NMDARs through the LIN-2/LIN-7/LIN-10 complex (Setou et al. 2000; Ho et al. 2003).

GRIP1 interacts with conventional kinesin (KIF5), linking it to AMPARs and directing KIF5 to dendrites (Setou et al. 2002). Because GRIP associates with EphB receptors and ephrin ligands, KIF5 may also transport these proteins in dendrites. Indeed, siRNA knockdown of GRIP1 causes a mislocalization of GluR2, EphB2 receptor, and KIF5 (Hoogenraad et al. 2005). These results suggest that GRIP1 acts as an adaptor protein that links KIF5 to EphB receptors and AMPARs.

The GRIP-interacting protein liprin- α also associates with KIF1A, another kinesin motor (Shin et al. 2003). The role for liprin- α in neuronal transport was demonstrated in a recent genetic study in *Drosophila*, in which liprin- α -deficient axons showed reduced anterograde transport but increased initiation of retrograde transport (Miller et al. 2005). The association of two different kinesin motors (KIF1A and KIF5) with AMPARs suggests redundancy in molecular transport mechanisms for physiologically important cargoes.

11

Conclusions

This review mainly described how PSD proteins coordinate the dynamic changes in the structure and function of excitatory synapses. Accumulating data indicate that apparently different aspects of synaptic functions are closely associated and may be regulated by common PSD proteins. For instance, regulation of spine morphogenesis is intimately associated with the regulation of synaptic strength and plasticity. Synaptic cell adhesion molecules may not only induce synapse formation but also promote spine morphogenesis and synaptic maturation. Another point to be considered is that the same PSD proteins may have different functions in different spatiotemporal contexts. For instance, synaptic scaffolds may function as motor receptors for the transport of specific cargoes during early stages of neuronal development, whereas they mainly participate in the regulation of receptor trafficking and recycling in mature neurons. Future studies may clarify these issues and increase our understanding of the molecular organization of the dynamic excitatory synapse.

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Molecular Organization and Assembly of the Central Inhibitory Postsynapse

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Abstract γ -Amino butyric acid type A (GABA_A) receptors are the major sites of fast synaptic inhibition in the brain. GABA_A receptors play an important role in regulating neuronal excitability and in addition have been implicated in numerous neurological disorders. In order to understand synaptic inhibition it is important to comprehend the cellular mechanisms, that neurons utilize to regulate the accumulation and regulation of GABA_A receptors at postsynaptic inhibitory specializations. Over the past decade a number of GABA_A receptor interacting proteins have been identified allowing us to further understand the trafficking, targeting and clustering of these receptors as well as the regulation of receptor stability. In the following review we examine the proteins identified as GABA_A receptor binding partners and other components of the inhibitory postsynaptic scaffold, and how they contribute to the construction of inhibitory synapses and the dynamic modulation of synaptic inhibition.

1 Introduction

Fast inhibitory neurotransmission in the adult central nervous system is mediated by the neurotransmitter GABA (γ -amino butyric acid) acting on GABA type A receptors. GABA_A receptors are the targets for various clinical drugs including benzodiazepines, barbiturates and anaesthetics. In addition they have been implicated in a number of neurological disorders including epilepsy (Olsen et al. 1999; Coulter 2001), anxiety (Malizia 1999), substance abuse (Morrow et al. 2001), Huntington's disease (Kunig et al. 2000), ischemia (Schwartz-Bloom and Sah 2001), Angelman's Syndrome (DeLorey et al. 1998) and schizophrenia (Lewis 2000; Nutt and Malizia 2001; Blum and Mann 2002).

Functional changes in the activity of ligand-gated ion channels are achieved by either regulating their activity, their cell surface expression levels or their localization within the plasma membrane. The regulation of inhibitory neurotransmission by phosphorylation of GABA_A receptors has been extensively reviewed elsewhere (Moss and Smart 2001; Brandon et al. 2002; Kittler and Moss 2003; Song et al. 2004). Therefore, here we will discuss how

recently identified GABA_A receptor-associated proteins regulate the cell surface dynamics of these receptors, their accumulation at inhibitory synapses, and the efficacy of synaptic inhibition.

1.1

GABA_A Receptor Structure

GABA_A receptors are chloride-permeable channels, which are members of the ligand gated ion channel superfamily. Members of this family are homologous in structure and include muscle and neuronal nicotinic acetylcholine receptors, glycine receptors and serotonin receptors (Schofield et al. 1987; Unwin 1993). GABA_A receptors are hetero-oligomeric pentamers composed of homologous subunits, which share a common structure encompassing a large extracellular amino terminus, four transmembrane (TM) domains and a large

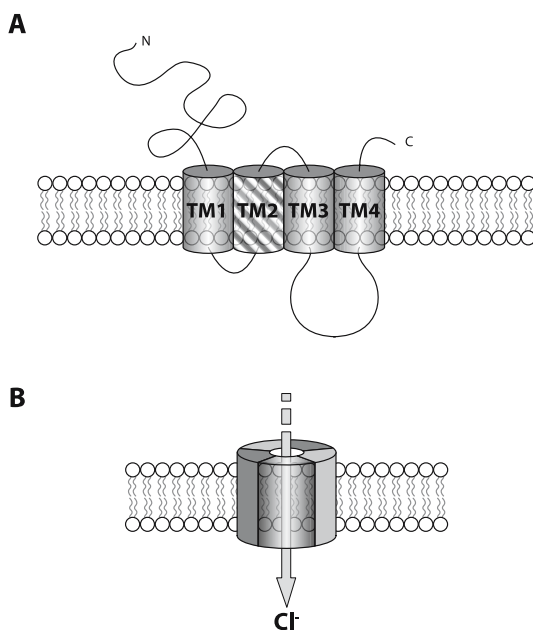


Fig. 1 GABA_A receptor structure. **a** GABA_A receptors are members of the ligand gated ion channel superfamily. Receptor subunits consist of four hydrophobic transmembrane domains (TM 1–4), where TM2 is believed to line the pore of the channel. The large extracellular N-terminus is the site for ligand binding as well as the site of action of various drugs. Each receptor subunit also contains a large intracellular domain between TM3 and TM4, which is the site for various protein interactions as well as the site for post-translational modifications that modulate receptor activity. **b** Five subunits from seven subunit subfamilies assemble to form a heteropentameric chloride permeable channel. Despite the extensive heterogeneity of GABA_A receptors most synaptic receptors are thought to consist of 2 α , 2 β and 1 γ subunit

intracellular loop between TM3 and TM4 (Fig. 1). In the mammalian nervous system, 16 genes encoding GABA_A receptor subunits have been identified. These genes can be divided into seven subunit subfamilies according to amino acid sequence homology: α (1–6), β (1–3), γ (1–3), δ , ε , π and θ . In addition many of these genes undergo alternative splicing, the most predominant of these being the γ 2 subunit, which exists in a short (γ 2S) and long (γ 2L) form. The large diversity of GABA_A receptor subunits generates the potential for a bewildering heterogeneity of receptor structure. However, studies on receptor assembly have revealed that only a limited number of receptor subunit combinations are likely to exist on the neuronal cell surface (Sieghart et al. 1999). With the exception of the β 1, β 3 and γ 2S subunits, expression of individual GABA_A receptor subunits in heterologous systems results in an accumulation in the endoplasmic reticulum (ER) and no cell surface expression (Connolly et al. 1999; Ebert et al. 1999). Homomeric channels formed from β 3 subunits are constitutively active (Krishek et al. 1996; Wooltorton et al. 1997) whereas homomeric β 1 receptors appear to be GABA gated depending on the species origin of the subunit being expressed (Krishek et al. 1996). On the other hand, expression of the γ 2S subunit alone does not result in the formation of functional channels, consistent with the suggestion that it can access the cell surface as a monomer (Connolly et al. 1999). The α and β subunits co-expressed in cultured cells result in the formation of functional GABA gated channels at the cell surface, however, these differ from most native receptors in that they lack benzodiazepine sensitivity (Angelotti and Macdonald 1993). Cells expressing α , β and γ subunits, conversely, form GABA gated, benzodiazepine-sensitive chloride channels (Angelotti and Macdonald 1993). A combination of immunocytochemical, pharmacological and electrophysiological studies are all in agreement that most GABA_A receptors expressed in the brain consist of two α , two β and one γ subunit (Chang et al. 1996; Tretter et al. 1997; Farrar et al. 1999; Knight et al. 2000) where the γ subunit can be replaced by δ , ε , or π depending on the cellular/subcellular localization of the receptor (Shivers et al. 1989; Davies et al. 1997; Neelands and Macdonald 1999).

1.2

GABA_A Receptor Localization within the Brain

GABA_A receptors with different subunit composition have been shown to have different pharmacological and physiological properties (Mohler et al. 2001; Rudolph et al. 2001; Mody and Pearce 2004; Farrant and Nusser 2005). In addition, a number of immunocytochemical studies have revealed differential subcellular targeting of GABA_A receptors depending on their subunit composition as well as differential expression throughout different brain regions. Furthermore, there is evidence to suggest that GABA_A receptor subunit expression varies in development as well as with disease (Laurie et al. 1992a; Cossart et al. 2005).

1.2.1

Regional Distribution of GABA_A Receptors

GABA_A receptors have ubiquitous expression in the brain, with $\alpha 1$, $\beta 2/3$, and $\gamma 2$ being the most abundant subunits (Laurie et al. 1992a; Wisden et al. 1992; Pirker et al. 2000). Whereas the $\alpha 1$ subunit is found to be present in most brain regions the $\alpha 4$ subunit is the least abundant isoform in the brain and appears to be restricted to the thalamus, the striatum and the molecular layer of the dentate gyrus (Wisden et al. 1992; Pirker et al. 2000). In contrast, $\alpha 5$ is found enriched in the CA1 region of the hippocampus whereas $\alpha 6$ is found almost exclusively in the granular layer of the cerebellum (Laurie et al. 1992b; Pirker et al. 2000).

All three β subunits are widely distributed in the brain although $\beta 1$ is expressed at much lower levels in comparison to $\beta 2$ and $\beta 3$ (Pirker et al. 2000). Some brain regions, however, appear to have higher concentrations of one subunit and consequently lower concentrations of other β subunits. For example, $\beta 2$ subunits are highly expressed in the thalamus in comparison to $\beta 1$ and $\beta 3$ subunits that showed little immunoreactivity in this region (Wisden et al. 1992; Pirker et al. 2000). In contrast, $\beta 3$ is found to be expressed at high levels in the striatum whereas $\beta 2$ is still present but at lower levels (Pirker et al. 2000). In addition, in the hippocampus $\beta 1$ and $\beta 3$ are found at higher concentrations than $\beta 2$ and are mainly found in principal neurons whereas $\beta 2$ shows stronger staining on interneurons (Pirker et al. 2000).

The gamma subunits are all found widely distributed with $\gamma 2$ being, by far, the most abundant one. The $\gamma 1$ subunit is found at higher concentrations in the pallidum and substantia nigra whereas $\gamma 3$ is diffusely distributed at low concentrations throughout the brain, although slightly more concentrated in the cerebral cortex (Laurie et al. 1992b; Wisden et al. 1992; Pirker et al. 2000). Despite being widely expressed, $\gamma 1$ and $\gamma 3$ do not substitute for the $\gamma 2$ subunit in $\gamma 2$ knockout mice, which show a lethal phenotype (Essrich et al. 1998).

Immunohistochemical and in situ hybridization studies are all in agreement that the δ subunit of GABA_A receptors is specifically concentrated in the granular layer of the cerebellum and in the thalamus, where it is thought to be assembled only in receptors that contain $\alpha 6$ and $\alpha 4$ subunits, respectively (Laurie et al. 1992b; Wisden et al. 1992; Pirker et al. 2000).

1.2.2

GABA_A Receptor Subcellular Localization

In addition to differential subunit expression throughout brain regions, immunocytochemical and immunogold electron microscopy studies have revealed that GABA_A receptor subunit composition varies between cell types and undergoes differential subcellular targeting.

Studies in cultured hippocampal neurons and cerebral slices have revealed that $\alpha 1$ – 3 and $\alpha 5$ subunit expression is located in the soma, dendrites and axons. The $\alpha 2$ subunit, however, is found to be concentrated in the axon initial segment (AIS) of the majority of cells, where it co-localizes with gephyrin indicating a synaptic localization (Nusser et al. 1996; Brunig et al. 2002). In contrast, $\alpha 1$ is found in somata and both proximal and distal dendrites, showing both diffuse and clustered staining. The $\alpha 1$ subunit was not found in the AIS on its own, but only in those neurons that also expressed $\alpha 2$, indicating that subunit composition of GABA_A receptors in the AIS consists of one copy of $\alpha 1$ and one of $\alpha 2$ (Brunig et al. 2002). Clusters of $\alpha 1$ are found to co-localize with gephyrin, whereas diffuse $\alpha 1$ staining indicates that a considerable amount of $\alpha 1$ containing receptors are found extrasynaptically (Brunig et al. 2002).

The subcellular targeting of $\alpha 3$ containing receptors appears to be dependent on the type of cell where it is expressed. In a subset of hippocampal cells, characterized by a round cell body and numerous short dendrites, $\alpha 3$ staining is generally diffuse and with no gephyrin co-localization although in the AIS large clusters can also be found (Brunig et al. 2002). In contrast, in pyramidal-like cells $\alpha 3$ was found in clusters at postsynaptic sites (Brunig et al. 2002). Staining of $\alpha 5$, on the other hand, was found to be diffuse in all hippocampal cells and never co-localized with gephyrin clusters (Brunig et al. 2002).

Pharmacological studies have revealed the importance of receptor composition in defining the biophysical properties of GABA_A receptors (reviewed in Farrant and Nusser 2005). The type of α subunit, for example, plays an important role in defining the sensitivity of the receptor to GABA and the deactivation rate of the receptor (Lavoie et al. 1997; Bohme et al. 2004; Farrant and Nusser 2005). In addition, the $\gamma 2$ and δ subunits can also play a role in modulating receptor sensitivity to GABA and deactivation, as well as being important in determining channel opening times (Fisher and Macdonald 1997; Haas and Macdonald 1999; Brown et al. 2002; Farrant and Nusser 2005). In the cerebellum, electrophysiological studies have identified two types of GABA_A receptor-mediated inhibition: a phasic and a tonic inhibition and this has been attributed to synaptic and extrasynaptic GABA_A receptors, respectively (Brickley et al. 1996, 1999). Immunocytochemical studies on electron microscopic sections confirmed that in cerebellar granule cells synaptic and extrasynaptic GABA_A receptor subunit composition differed. Synapses in cerebellar granule neurons were found to be positive for $\alpha 1$, $\alpha 6$, $\beta 2/3$ and $\gamma 2$ immunogold staining whereas the δ subunit was found specifically in extrasynaptic membranes associated with $\alpha 6$ subunits (Nusser et al. 1998a). Furthermore, consequent electrophysiological studies have confirmed a role for the $\gamma 2$ subunit in mediating phasic inhibition and a role for the δ subunit in mediating tonic inhibition (Brickley et al. 1999; Brickley et al. 2001; Stell et al. 2003).

2

Formation and Maintenance of Inhibitory Synapses

A number of proteins have been implicated in regulating the clustering and targeting of GABA_A receptors to postsynaptic inhibitory specializations. Recent findings have implicated neuroligins and neurexins as key players in both excitatory and inhibitory synapse formation, reviewed in (Washbourne et al. 2004; Levinson and El-Husseini 2005).

Neuroligins are postsynaptic transmembrane proteins encoded by four different genes. All four neuroligins (NL 1–4) have been shown to bind the presynaptic transmembrane protein β -neurexin but not its splice isoform α -neurexin in vitro and in vivo (Ichtchenko et al. 1996; Dean et al. 2003). An elegant study by Scheiffele et al. demonstrated that the interaction between neuroligins and β -neurexin played an important role in triggering excitatory synaptogenesis in non-neuronal cells (Scheiffele et al. 2000; Fu et al. 2003). Furthermore, neuroligins have been shown to interact with PSD-95 and this interaction has been suggested to provide a link for the recruitment of neurotransmitter receptors and accessory proteins to postsynaptic sites (Washbourne et al. 2004; Chih et al. 2005). Interestingly, suppression of individual neuroligins using small-hairpin RNAs (shRNA) results in reduced synapse formation (Chih et al. 2005), whereas NL1 and NL2 knockout mice are both viable and behaviourally normal (Song et al. 1999; Varoqueaux et al. 2004). The basis for this discrepancy between in vitro and in vivo observations will have to be revealed in future.

Until recently, neuroligins have been shown to play a role in excitatory synaptogenesis. However, it is now evident that whereas NL1 is found at excitatory synapses, NL2 is exclusively found at inhibitory synapses where it co-localizes with GABA_A receptors (Varoqueaux et al. 2004; Chih et al. 2005; Levinson et al. 2005). Using a number of neuron-fibroblast co-culture assays, Craig and colleagues demonstrated that expression of β -neurexin is able to induce clusters of the inhibitory postsynaptic marker gephyrin. In the same study, expression of NL2 in fibroblasts was able to induce the clustering of GAD-65 positive GABAergic synaptic vesicles in opposing terminals (Graf et al. 2004).

Despite their subcellular distribution, overexpression of NL1, 2 or 3 can induce the formation of both excitatory and inhibitory synapses (Prange et al. 2004; Chih et al. 2005; Levinson et al. 2005). Interestingly, expression of NL1 together with PSD-95 results in the formation of new excitatory but not inhibitory synapses (Prange et al. 2004). Furthermore overexpression of PSD-95 causes a redistribution of NL2 to excitatory synapses, presumably by an interaction between PSD-95 and the PDZ domain in NL2 (Levinson et al. 2005). Overall these data suggest that the neuroligin–neurexin complex plays an important role in excitatory and inhibitory synaptogenesis, although it appears that, in addition, postsynaptic scaffolding proteins may be required for con-

trolling the balance between excitatory and inhibitory synapses (Levinson and El-Husseini 2005).

GABA_A receptor clustering at postsynaptic sites is thought to be dependent on the 93 kDa protein gephyrin. Gephyrin was initially identified as a protein involved in mediating glycine receptor clustering via a direct interaction with the intracellular domain of the β subunit of these receptors (Pfeiffer et al. 1982; Meyer et al. 1995; Kneussel et al. 1999a). Gephyrin is specifically localized to glycinergic synapses in spinal cord, retina and hippocampal neurons. Depletion of gephyrin expression using antisense oligonucleotides inhibited synaptic clustering of glycine receptors (Kirsch et al. 1993) and neuronal cultures from gephyrin knockout mice show diffuse staining of glycine receptors confirming the importance of gephyrin in glycine receptor clustering (Feng et al. 1998).

In hippocampal and cortical neurons, where GABA is the major inhibitory neurotransmitter and glycine receptor expression is relatively low, gephyrin was found to be expressed in clusters at postsynaptic sites where it colocalized with GABA_A receptors (Craig et al. 1996; Essrich et al. 1998). Furthermore a study by Essrich and colleagues showed that in cultures from $\gamma 2$ knockout mice there are no GABA_A receptors accumulated at postsynaptic sites and this loss is parallel to a loss in gephyrin clusters (Essrich et al. 1998). In addition hippocampal neurons treated with gephyrin antisense oligonucleotides, hippocampal cultures, and spinal cord sections from gephyrin knockout mice all show a loss of $\alpha 2$ and $\gamma 2$ clusters (Essrich et al. 1998; Kneussel et al. 1999b). However, the overall levels of $\alpha 2$ and $\gamma 2$ receptor subunits in gephyrin knockout mice do not differ from control animals but are accumulated intracellularly, suggesting that the clustering of GABA_A receptors at postsynaptic sites requires an interaction between gephyrin and $\gamma 2$ subunits (Essrich et al. 1998; Kneussel et al. 1999b). However, despite these observations it has not so far been possible to show a direct interaction between gephyrin and GABA_A receptors.

Gephyrin binds tubulin via a region with high homology to the microtubule-associated proteins MAP-2 and Tau (Ramming et al. 2000). In addition, the N-terminal domain of gephyrin has the ability to trimerize whereas the C-terminal domain can form dimers (Schwarz et al. 2001). These observations suggest that gephyrin can assemble as a lattice and act as a scaffold at postsynaptic sites. Recent observations from gephyrin knockout mice are in agreement with gephyrin acting as a scaffold rather than as a protein required for clustering at GABAergic synapses. Hippocampal cultures from gephyrin knockout mice only showed a 23% reduction in the amplitude of GABA currents compared to wild type neurons (Levi et al. 2004). In addition, synaptic aggregation of $\alpha 1$ containing GABA_A receptors was unaffected by gephyrin depletion, whereas $\alpha 2$ containing receptor levels at the cell surface were reduced by 65%, and $\gamma 2$ containing receptors by 43% (Levi et al. 2004). These results were consistent with previous observations

in immature neurons where GABA_A receptor clusters form before gephyrin can be detected at postsynaptic sites (Dumoulin et al. 2000; Danglot et al. 2003). Together, these results implicate a role in the clustering of a subset of GABA_A receptors, however, gephyrin co-localizes with all GABA_A receptors independent of subunit composition. A recent study looked at the cell surface dynamics of GABA_A receptors in the presence and absence of gephyrin (Jacob et al. 2005). Using FRAP (fluorescence recovery after photobleaching) and RNAi technology, they observed that in the absence of gephyrin clustered GABA_A receptors were three times more motile than those in control neurons, indicating that gephyrin plays a role in enhancing the confinement of GABA_A receptors at synaptic sites (Jacob et al. 2005). This suggests that gephyrin, much like PSD-95 at excitatory synapses, acts as a scaffold and aids in the stabilization of previously clustered GABA_A receptors.

Another protein found highly concentrated at inhibitory synapses is dystrophin (Knuesel et al. 1999). In brain slices of 2–3 month old rats, dystrophin co-localizes with $\alpha 2$ and $\gamma 2$ GABA_A receptor subunit clusters found in the soma and dendrites of pyramidal cells as well as $\alpha 1$ and $\gamma 2$ clusters in the soma and proximal dendrites of Purkinje cells (Knuesel et al. 1999). Interestingly, not all GABA_A receptor clusters are found co-localized with dystrophin, indicating that dystrophin is found at a subset of inhibitory synapses (Knuesel et al. 1999). In brain sections from *mdx* mutant mice, which lack dystrophin, GABA_A receptor clusters in pyramidal and Purkinje cells are reduced in size and number whereas gephyrin clusters appear unaltered (Knuesel et al. 1999). Dystrophin is found associated with α -dystroglycan and β -dystroglycan to form the dystrophin-associated glycoprotein complex (DGC). Analysis of both α - and β -dystroglycan localization in cultured hippocampal neurons revealed that, like dystrophin, dystroglycan is found at a subset of GABAergic terminals, although this co-localization was only evident in late stages of development (Levi et al. 2002). In cultured hippocampal neurons from dystroglycan conditional knockout mice, there was no evidence of dystrophin accumulation yet GABA_A receptor and gephyrin synaptic accumulation was unaltered (Levi et al. 2002). Together, these results indicate that the DGC is not required for GABA_A receptor clustering, but might play a stabilizing role for the receptor at inhibitory synapses.

3

Trafficking and Targeting of GABA_A Receptors

Given the selective subcellular expression of GABA_A receptors it is clear that the trafficking, targeting and clustering of these receptors is a tightly controlled and regulated process. A number of GABA_A receptor-associated proteins have been recently identified and implicated in these processes (Table 1);

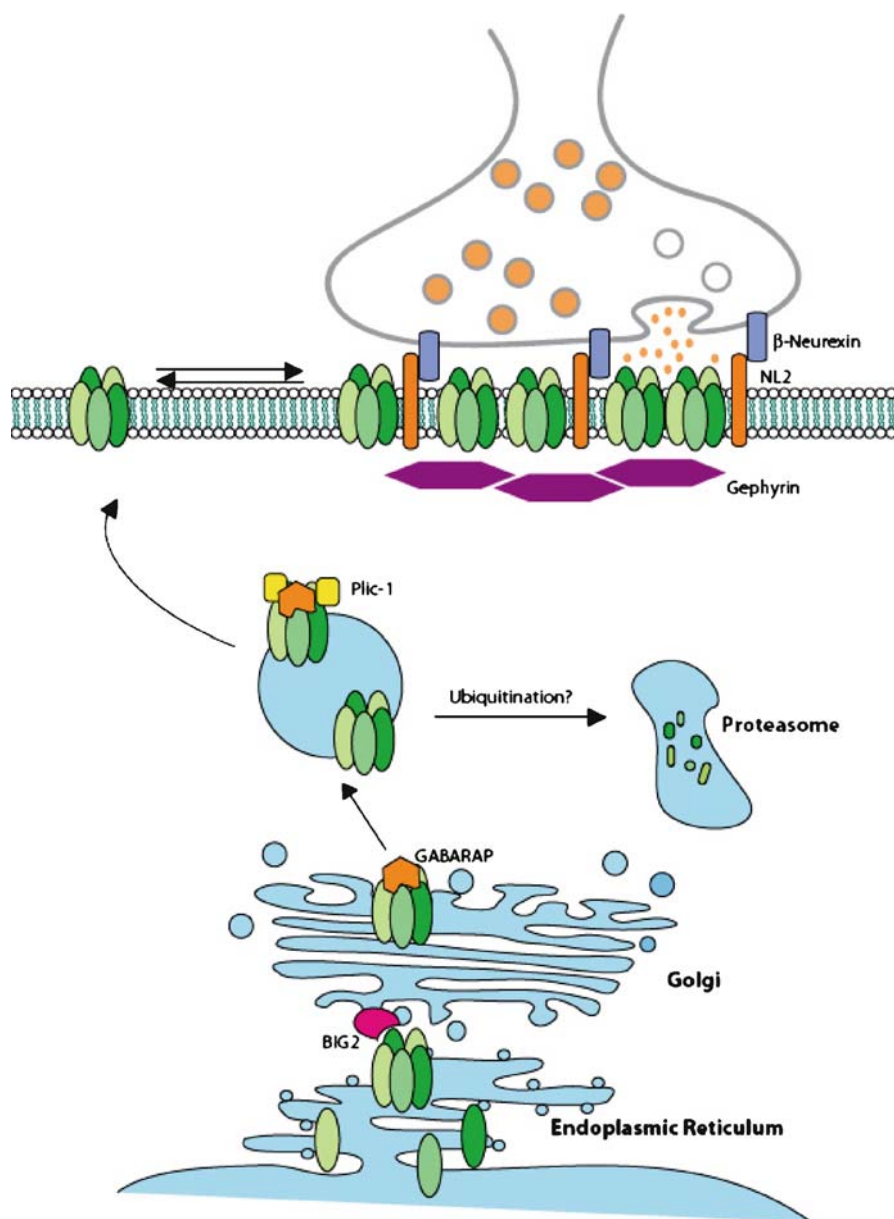
Table 1 Proteins associated with GABA_A receptors

Protein	Cellular localization	Interaction with GABA _A R	Putative function	Refs.
NL2	Synapses	No	Synapse formation	Graf et al. 2004; Varoqueaux et al. 2004; Chih et al. 2005
Gephyrin	Synapses	No	GABA _A R clustering/scaffold	Craig et al. 1996; Essrich et al. 1998; Levi et al. 2004
Dystrophin	Synapses	No	Stabilization	Knuesel et al. 1999
GABARAP	Golgi	$\gamma 2$ subunit	Trafficking	Wang et al. 1999; Kittler et al. 2001; Leil et al. 2004; Chen et al. 2005
PRIP-1	Synapses	$\beta 3$ subunit	Regulation of GABA _A R phosphorylation state/trafficking	Kanematsu et al. 2002; Terunuma et al. 2004
Plic-1	Intracellular compartments	α and β subunits	Surface number regulation	Bedford et al. 2001
AP2	Clathrin-coated pits	β and γ subunits	Endocytosis of GABA _A Rs	Kittler et al. 2000
HAP1A	Endosomes	β subunits	Post-endocytic sorting of GABA _A Rs	Kittler et al. 2004
BIG-2	Intracellular compartments	β subunits	Trafficking	Charych et al. 2004
GODZ	Golgi	γ subunits	Palmitoylation of γ subunit	Keller et al. 2004
GRIF-1	Intracellular	$\beta 2$ subunit	Unknown	Beck et al. 2002
gC1qR	Intracellular compartments/mitochondria	$\beta 3$ subunit	Unknown	Schaerer et al. 2001

those that have been identified to regulate receptor cell surface expression are discussed below (Fig. 2).

Evidence from neuronal cultures from mice deficient in $\gamma 2$ subunit expression revealed that although this subunit is not essential for assembly and membrane expression it is essential for the synaptic localization, clustering and function of GABA_A receptors. Using the $\gamma 2$ subunit as the bait in a yeast two-hybrid screen allowed for the identification of a 17 kDa GABA_A receptor-associated protein (GABARAP) (Wang et al. 1999). GABARAP has been shown to bind γ subunits both in vitro and in vivo. Although it is not found at inhibitory synapses it appears to co-localize with GABA_A receptors in intracellular compartments resembling the Golgi and subsynaptic cisternae (Wang et al. 1999; Kittler et al. 2001). Sequence analysis of GABARAP re-

vealed similarity to the small microtubule binding proteins GATE-16 and the light chain-3 (LC3) subunit of MAP-1A and 1B. Further analysis has shown that GABARAP is able to bind microtubules as well as *N*-ethylmaleimide sensitive factor (NSF), supporting a role for GABARAP in GABA_A receptor intracellular transport (Wang and Olsen 2000; Kittler et al. 2001). A functional role



for GABARAP in the trafficking of GABA_A receptors to the plasma membrane was recently confirmed in a set of immunochemical and electrophysiological studies where co-expression of GABARAP and GABA_A receptors in COS-7 cells resulted in an increase in the levels of GABA_A receptors expressed at the cell surface (Leil et al. 2004) as well as an increase of GABA-mediated currents in oocytes (Chen et al. 2005).

More recently, a yeast two-hybrid screen identified GRIP-1 as a binding partner for GABARAP (Kittler et al. 2004a). GRIP-1 was found to interact with GABARAP and, in addition, immunostaining in cultured hippocampal neurons showed extensive co-localization with the $\gamma 2$ subunit of GABA_A receptors (Kittler et al. 2004a). GRIP-1, previously identified as a glutamate receptor interacting protein, is a 7 PDZ domain protein that plays an essential role in AMPA receptor clustering (Dong et al. 1997). GRIP-1 and the 4-PDZ domain splice form GRIP-1c (PDZ 4-7) have also been observed at inhibitory synapses in cultured neurons and in brain slices (Burette et al. 1999; Dong et al. 1999; Wyszynski et al. 1999; Charych et al. 2004b; Li et al. 2005). The role of GRIP-1 at inhibitory synapses remains unknown, although its ability to interact with GABARAP suggest that it may be involved in the synaptogenesis of inhibitory synapses or in the regulation of GABA_A receptor function (Kittler et al. 2004a).

Another recently identified GABARAP-associated protein is the phospholipase-C related inactive protein type 1 (PRIP-1) (Kanematsu et al. 2002). PRIP-1 does not associate with the $\gamma 2$ subunit of GABA_A receptors but appears to compete with this for GABARAP binding (Kanematsu et al. 2002). Furthermore, PRIP-1 knockout mice showed an impairment in GABA_A receptor modulation by $\gamma 2$ selective pharmacological agents such as benzodiazepines and zinc (Kanematsu et al. 2002). These findings suggested that PRIP-1 might play a role in the regulation of GABA_A receptor trafficking by GABARAP, ensuring that only mature $\alpha\beta\gamma$ receptors are delivered to the plasma membrane (Kanematsu et al. 2002). Further studies with PRIP-1 and GABA_A receptors have revealed a different model by which PRIP-1 modulates GABA_A receptor function in a manner that is independent of GABARAP (Terunuma et al. 2004). PRIP-1 has been shown to bind the cata-

- ◀ **Fig. 2** Trafficking of GABA_A receptors. GABA_A receptors are assembled in the endoplasmic reticulum and exit into the Golgi complex is thought to be mediated by aid of the guanine exchange factor BIG2. The microtubule binding protein GABARAP associates with the $\gamma 2$ subunit of GABA_A receptors at the Golgi and aids in the trafficking to the plasma membrane. In addition, the ubiquitin like protein Plic-1 is localized in intracellular compartments where it binds α and β subunits. Binding of Plic-1 to the GABA_A receptor is thought to regulate membrane targeting by preventing degradation in the proteasome. GABA_A receptors are thought to be inserted into the plasma membrane at extrasynaptic sites where they are allowed to diffuse into synaptic sites and are clustered there by an unknown interaction with the scaffolding protein Gephyrin

lytic subunit of protein phosphatase 1 α (PP1 α) (Yoshimura et al. 2001) and the GABA_A receptor β 3 subunit (Terunuma et al. 2004). Given the functional modulation of GABA_A receptors by phosphorylation of GABA_A receptor β 3 subunits by PKA, Terunuma et al. investigated the role of PRIP-1 in the regulation of the phosphorylation state of GABA_A receptors. Indeed PP1a activity was significantly enhanced in PRIP-1 knockout mice resulting in a reduction of β 3 phosphorylation by PKA (Terunuma et al. 2004). Furthermore PRIP-1 is also a substrate for PKA and in its phosphorylated state PRIP-1 dissociates from PP1a allowing for phosphatase activity (Terunuma et al. 2004). The authors propose a model where PRIP-1 regulates GABA_A receptor phosphorylation and thus synaptic inhibition (Terunuma et al. 2004).

GABA_A receptors also interact with the ubiquitin-related protein Plic-1. Plic-1 is a 67 kDa protein with a ubiquitin-like N-terminus and a carboxy-terminal ubiquitin-associated domain (UBA) (Wu et al. 1999; Kleijnen et al. 2000). Plic proteins have been shown to bind the proteasome and ubiquitin ligases and, hence, are thought to regulate ubiquitin-dependent protein degradation in the proteasome by interfering with the ubiquitination machinery (Kleijnen et al. 2000). The interaction between Plic-1 and GABA_A receptors was originally identified from a yeast two-hybrid screen using the intracellular loop of the α 1 GABA_A receptor subunit as the bait (Bedford et al. 2001). Further studies using yeast two-hybrid screens and GST pull-down assays revealed an interaction between Plic-1 and all α (1–6) and β (1–3) subunits of GABA_A receptors, indicating that Plic-1 function may be relevant for the majority of receptor subtypes expressed in the brain (Bedford et al. 2001). Immunocytochemical studies found Plic-1 to be mainly expressed intracellularly in the cell body and dendritic axonal processes, consistent with its proteasome binding properties (Kleijnen et al. 2000; Bedford et al. 2001). Despite its subcellular localization, a significant number of GABA_A receptors were found to co-localize with Plic-1, immunostaining beneath the plasma membrane and synaptic sites (Bedford et al. 2001). Interestingly, binding to the intracellular loops of α and β subunits is mediated by the UBA domain of Plic-1. Functional studies revealed that blockade of this interaction resulted in reduced cell surface expression of GABA_A receptors as demonstrated by electrophysiological studies in both HEK293 cells and hippocampal slices (Bedford et al. 2001). Consistent with these results, overexpression of Plic-1 in recombinant systems caused an increase in GABA_A receptors expressed at the cell surface without affecting internalization rates (Bedford et al. 2001). Although the mechanisms by which Plic-1 modulates GABA_A receptor function remain unknown, these results suggest that Plic-1 regulates GABA_A receptor cell surface numbers by facilitating membrane insertion, presumably by inhibiting their degradation by the proteasome.

The GABA_A receptor γ 2 subunit, which is essential for the synaptic clustering of GABA_A receptors (Essrich et al. 1998), has recently been shown

to be a substrate for palmitoylation (Keller et al. 2004; Rathenberg et al. 2004). Palmitoylation is a post-translational modification that involves attachment of the fatty acid palmitate to cysteine residues and has been shown to be involved in the membrane targeting and subcellular trafficking of various proteins, including the neuronal scaffold proteins PSD-95 and GRIP (DeSouza et al. 2002; El-Husseini Ael et al. 2002; Smotrys and Linder 2004). Palmitoylation of the $\gamma 2$ subunit of GABA_A receptors is dependent on the palmitoylase transferase GODZ (Golgi-specific DHHC zinc finger domain protein) (Keller et al. 2004). GODZ was found to interact with a 14-amino acid cysteine-rich domain conserved in the intracellular loops of all $\gamma(1-3)$ subunits in a SOS-recruitment yeast two-hybrid assay, but not by pull-down assays, suggesting that the interaction between GODZ and γ subunits is transient (Keller et al. 2004). Similarly GODZ was found to be localized in the Golgi where there was partial overlap with the $\gamma 2$ subunit in HEK293 cells but not in neurons (Keller et al. 2004). Interestingly, mutating the cysteine residues within the intracellular loop of the $\gamma 2$ subunit resulted in a loss of GABA_A receptor clusters at the cell surface, as did treatment with the palmitoylation inhibitor 2-BrP (Rathenberg et al. 2004). From these studies it is evident that GABA_A receptor palmitoylation plays a critical role in the trafficking and clustering of GABA_A receptors, although the exact mechanisms by which this is achieved remain unknown.

The brefeldin A-inhibited GDP/GTP exchange factor 2 (BIG2) has also been identified in a yeast two-hybrid screen as a GABA_A receptor β subunit interacting protein (Charych et al. 2004a). BIG2 is mainly found concentrated in the trans-Golgi network although a small proportion is found in vesicle-like structures along dendrites and near postsynaptic sites (Charych et al. 2004a). BIG2 has previously been implicated in vesicular transport by its ability to catalyse the GDP-GTP exchange on ARF GTPases. Interestingly, co-expression of BIG2 with the GABA_A receptor $\beta 3$ subunit results in an increase in $\beta 3$ exit from the ER (Charych et al. 2004a), suggesting that BIG2 is involved in the post-Golgi vesicular trafficking of GABA_A receptors.

Other GABA_A receptor interacting proteins have been identified including GRIF-1 (Beck et al. 2002) and gC1qR (Schaerer et al. 2001). These proteins, however, are not enriched at synapses and their functional significance remains to be established.

4

Receptor Stability

Regulating the number of GABA_A receptors at the cell surface is an important mechanism for regulating the strength of synaptic inhibition (Nusser et al. 1997, 1998b; Kittler et al. 2000; Kittler and Moss 2003). Therefore, a significant

amount of research has focussed on detailing the mechanisms that control the stability of cell surface receptor populations and their accumulation at inhibitory synapses.

Studies on both glycine and AMPA receptors using single particle tracking have revealed that there are significant rates of exchange between synaptic and extrasynaptic pools of these ion channels and that synaptic receptors exhibit reduced rates of lateral mobility compared to their extrasynaptic counterparts (Choquet and Triller 2003; Dahan et al. 2003; Tardin et al. 2003). More recently a study by Thomas et al. (2005) used a novel electrophysiological tagging method to examine the lateral movement of GABA_A receptors in cultured hippocampal neurons (Thomas et al. 2005). Their results demonstrate that in common with glycine and AMPA receptors, GABA_A receptors are dynamic along the plasma membrane and can readily diffuse in and out of synaptic sites, providing a molecular mechanism to regulate the efficacy of synaptic inhibition (Thomas et al. 2005). Another recent study is also in agreement with the ability of GABA_A receptors to diffuse along the plasma membrane. In addition, this study revealed that extrasynaptic receptors are more mobile than their synaptic counterparts and that this difference in motility is dependent upon the postsynaptic protein gephyrin (Jacob et al. 2005).

In addition to the ability to undergo lateral diffusion along the plasma membrane, GABA_A receptors have been shown to undergo extensive endocytosis in both heterologous and neuronal systems. Furthermore, a number of associated proteins have been identified as playing a key role in regulating GABA_A receptor internalization and post-endocytic sorting.

GABA_A receptor internalization is blocked by hypertonic sucrose in both heterologous recombinant systems and in cultured hippocampal neurons (Kittler et al. 2000), conditions which have been shown to impair clathrin function (Heuser and Anderson 1989). In addition, blocking dynamin function by using a peptide that disrupts the association between dynamin and amphiphysin, or by overexpression of the catalytically inactive dynamin K44A mutant, also results in a reduction of GABA_A receptor internalization (Kittler et al. 2000; Herring et al. 2003; van Rijnsoever et al. 2005). Together, these suggest that GABA_A receptors are internalized via clathrin-mediated endocytosis (Fig. 3). Moreover, both β and γ subunits have been shown to bind the clathrin adaptor protein complex AP2, an integral member of the clathrin endocytosis machinery (Kittler et al. 2000). In addition, a form of clathrin-independent GABA_A receptor endocytosis has been reported in HEK293 cells although there is not yet any evidence that this mechanism also occurs in neurons (Cinar and Barnes 2001).

A study using recombinant receptors in HEK293 cells revealed the importance of a dileucine motif present in all three β subunit isoforms for the internalization of GABA_A receptors (Herring et al. 2003). In addition, a novel AP2 binding motif has recently been identified in all β subunits of the GABA_A

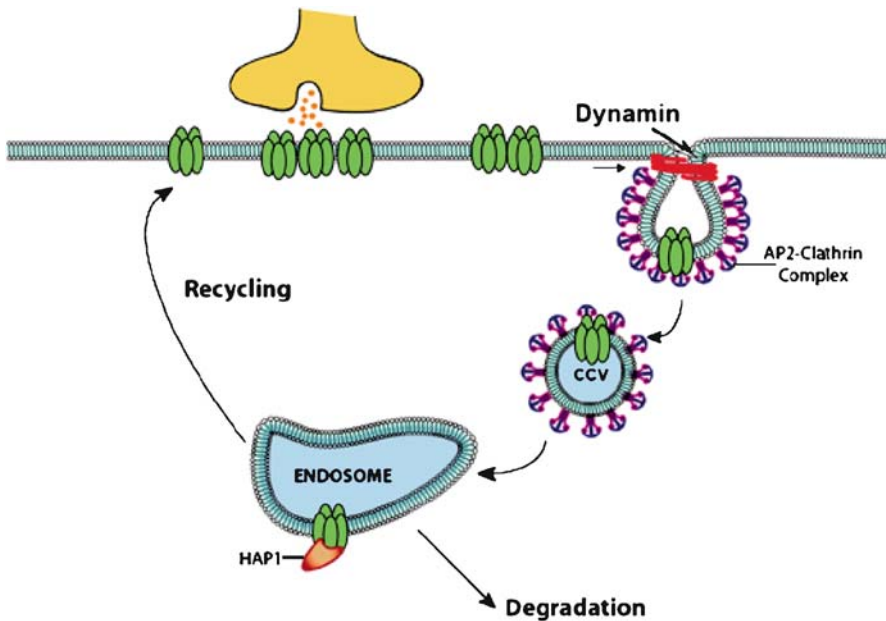


Fig. 3 Endocytosis of GABA_A receptors. GABA_A receptors have been shown to undergo constitutive clathrin mediated endocytosis. The intracellular loops of β and γ subunits are known to interact with the clathrin adaptor protein AP2. Upon internalization GABA_A receptors are transported to sorting endosomes where they can be sorted for either recycling back to the plasma membrane or for degradation. An interaction between β subunits and the endosomal protein HAP1 is thought to facilitate the recycling of receptors back to the plasma membrane

receptor (Kittler et al. 2005). Interestingly, this region includes the major sites of serine phosphorylation within β subunits and will only bind AP2 when in a dephosphorylated state (Kittler et al. 2005). These results provide direct evidence for the importance of phosphorylation in regulating receptor stability at the cell surface.

The physiological significance of modulating GABA_A receptor internalization has been illustrated in both heterologous expression systems and cultured neuronal cells. Blocking dynamin function, and hence endocytosis, resulted in an increase in amplitude of inhibitory postsynaptic currents (IPSCs) consistent with the idea of an increase in receptors at the cell surface (Kittler et al. 2000).

Quantitative measurements of GABA_A receptor endocytosis have revealed that approximately 25% of the total cell surface population of $\beta 3$ subunit-containing receptors are internalized within 30 min (Kittler et al. 2004b). Given this high rate of internalization the endocytic fate of internalized receptors is likely to play a critical role in regulating receptor cell surface

expression levels. It is emerging that internalized GABA_A receptors can be sorted for two endocytic fates. The majority of internalized receptors constitutively recycle back to the plasma membrane over short time frames (tens of minutes) but over longer time periods (6 h) 25% of surface receptors are degraded upon internalization (Kittler et al. 2004b). Furthermore, internalized receptors have been shown to localize to a subsynaptic pool associated with postsynaptic proteins such as gephyrin (van Rijnsoever et al. 2005). Recently huntingtin-associated protein-1 (HAP1) has been identified as a GABA_A receptor-associated protein (Kittler et al. 2004b). HAP1 binds the intracellular loop of β subunits in vitro and in vivo. Overexpression of HAP1 in cortical neurons results in a decrease of GABA_A receptor degradation and consequently an increase in GABA_A receptor recycling (Kittler et al. 2004b). These results identify HAP1 as a regulator of GABA_A receptor endocytic sorting (Fig. 3).

5

Concluding Remarks

GABA_A receptors are critical mediators of fast synaptic inhibition and drug targets. Therefore, comprehending the cellular mechanisms neurons utilize to regulate their cell surface stability and accumulation at inhibitory synapses is of fundamental importance.

Accumulating experimental evidence has begun to illustrate that GABA_A receptors are dynamic entities on the neuronal surface, and that these proteins show significant levels of both endocytosis and exocytosis. These processes can be regulated by receptor-associated proteins (Table 1) and have been shown to regulate the efficacy of synaptic inhibition. Moreover, the mechanisms that regulate the clustering and accumulation of GABA_A receptors at synaptic sites are also being actively pursued. It will be important to assess the role that these putative regulatory processes play in models of synaptic plasticity, such as rebound potentiation in the cerebellum, in which alterations in GABA_A receptor cell surface number are believed to be of significance.

Finally there is a growing list of CNS pathologies such as Status epilepticus, where altered GABA_A receptor functional expression is of major significance (Coulter 2001). Therefore, assessing the physiological and pathological role of GABA_A receptor membrane trafficking is an active area of current investigation.

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Molecular Organization and Assembly of the Presynaptic Active Zone of Neurotransmitter Release

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Abstract At chemical synapses, neurotransmitter is released at a restricted region of the presynaptic plasma membrane, called the active zone. At the active zone, a matrix of proteins is assembled, which is termed the presynaptic grid or cytomatrix at the active zone (CAZ). Components of the CAZ are thought to localize and organize the synaptic vesicle cycle, a series of membrane trafficking events underlying regulated neurotransmitter exocytosis. This review is focused on a set of specific proteins involved in the structural and functional organization of the CAZ. These include the multi-domain Rab3-effector proteins RIM1 α and RIM2 α ; Bassoon and Piccolo, two multi-domain CAZ scaffolding proteins of enormous size; as well as members of the CAST/ERC family of CAZ-specific structural proteins. Studies on ribbon synapses of retinal photoreceptor cells have fostered understanding the molecular design of the CAZ. In addition, the analysis of the delivery pathways for Bassoon and Piccolo to presynaptic sites during development has produced new insights into assembly mechanisms of brain synapses during development. Based on these studies, the active zone transport vesicle hypothesis was formulated, which postulates that active zones, at least in part, are pre-assembled in neuronal cell bodies and transported as so-called Piccolo-Bassoon transport vesicles (PTVs) to sites of synaptogenesis. Several PTVs can fuse on demand with the presynaptic membrane to rapidly form an active zone.

Abbreviations

C1 domain	conserved domain 1 of protein kinase C
C2 domain	conserved domain 2 of protein kinase C
CAST	CAZ-associated structural protein
CAZ	cytomatrix assembled at the active zone
CtBP	C-terminal binding protein (or connected to Bassoon and Piccolo)
DAG	diacylglycerol
ERC	ELKS-Rab6-interacting molecule-CAST
GIT	protein interacting with G-protein-coupled receptor kinases
PBH domain	Piccolo-Bassoon homology domain
PDZ	PSD-95-Discs large-ZO-1 domain
PSD	postsynaptic density
PTV	Piccolo-Bassoon transport vesicle
RIM	Rab3-interacting molecule

RIM-BP RIM-binding protein
SV synaptic vesicle

1

Introduction

Chemical synapses are important sites for intercellular communication. They constitute asymmetric cell-cell contact sites between a presynaptic and a postsynaptic cell devised to translate presynaptic electrical signals into chemical signals in the form of exocytosed neurotransmitter. This messenger can be detected by the postsynaptic cell and transformed back into an appropriate postsynaptic signal. The presynaptic active zone is the restricted area of the presynaptic plasma membrane, at which synaptic vesicles can fuse and release their neurotransmitter content in a regulated (Ca^{2+} -dependent) manner. The active zone is aligned precisely with the region of postsynaptic membrane that harbors the neurotransmitter reception apparatus and is delineated by the postsynaptic density (PSD). As defined here, the active zone consists of two major parts, the active zone plasma membrane and the associated cytoskeletal matrix, which is called cytomatrix assembled at the active zone (CAZ) or presynaptic grid (Dresbach et al. 2001; Gundelfinger et al. 2006). In this review, we will discuss recent discoveries concerning the molecular organization of the active zone and its developmental assembly during the major period of synaptogenesis during brain development.

2

General Aspects of Structural and Functional Organization of the Active Zone

2.1

Neurotransmitter Release

At the active zone, a series of membrane trafficking events underlies regulated exocytosis of neurotransmitter from synaptic vesicles (SV) and the retrieval and recycling of vesicular membranes: the SV cycle (Sudhof 1995, 2004). SV that are tethered to the active zonal membrane have to undergo a priming process to become fusion competent, i.e. to become responsive to influx of Ca^{2+} through voltage-gated Ca^{2+} channels located in the active zone membrane. Several Proteins, including Ca^{2+} and diacylglycerol (DAG)-regulated UNC13/Munc13 proteins, SM (Sec1/Munc18-like) proteins and RIM1/UNC10, are involved in this priming process (Rosenmund et al. 2003, see below). Upon membrane depolarization by action potentials, Ca^{2+} channels open

and a Ca^{2+} -sensing machinery detects Ca^{2+} ions entering the cell. Synaptotagmins are the major presynaptic Ca^{2+} sensors (Chapman 2002). However, other proteins, such as complexins, are also thought to convey the Ca^{2+} signal to the fusion machinery (Reim et al. 2001). The actual fusion process is mediated via SNAREs, including synaptobrevins/VAMPs as vesicular SNARE proteins and syntaxin and SNAP-25 as target membrane SNAREs (for review, see Jahn et al. 2003). After membrane fusion, SV may either fully collapse into the active zone membrane or release their neurotransmitter content through a fusion pore. In the latter case, the vesicle may be rapidly retrieved and re-filled in a “kiss-and-run” or “kiss-and-stay” mechanisms (Gundelfinger et al. 2003; Sudhof 2004). In the former case, SV vesicles are retrieved by clathrin-mediated endocytosis in the periactional zone area that surrounds the active zone. Exocytosis and endocytosis are thought to be spatially and temporally tightly linked (Dresbach et al. 2001; Gundelfinger et al. 2003; Murthy and De Camilli 2003).

Recycling and refilling processes are thought to occur somewhat remote from the active zonal membrane (Sudhof 1995, 2004). Depending on their functional state, different pools of SV can be defined: the cycling pool of vesicles, which includes vesicles that are docked and immediately available for release and those that are rapidly recycling and reused, and the reserve or resting pool of vesicles. The latter pool includes SV that are inactive and are recruited only after massive stimulation (Sudhof 2000, 2004).

2.2

Ultrastructural Organization of the Active Zone

The presynaptic active zone is precisely aligned with the postsynaptic neurotransmitter reception apparatus defined by the PSD. At the ultrastructural level, both sides of the synaptic contact site appear as electron-dense structures that are tightly associated with the plasma membrane. In the electron microscope, the CAZ or presynaptic grid appears as a more or less regular array of electron-dense cone-shaped particles, which extend ~ 50 nm into the cytoplasm (Gray 1963; Pfenninger et al. 1972; Phillips et al. 2001). A meshwork of cytoskeletal filaments connects the 50-nm pyramid-shaped particles; in addition filamentous strands extend deeply from the active zone plasma membrane into the presynaptic bouton. At the frog neuromuscular junction, which has been studied by electron microscope tomography, active zone material extends 50–75 nm into the cytoplasm of the terminal and has a regularly arranged ultrastructure consisting of “pegs”, “ribs” and “beams” (Harlow et al. 2001).

The CAZ is thought to define and organize neurotransmitter release sites, keeping them aligned with the PSD. CAZ components mediate the replacement of exocytosed SV (Dresbach et al. 2001; Garner et al. 2002). Moreover, the CAZ may play an important role in the spatial and temporal coordi-

nation of exocytic and endocytic events at the active zone (Gundelfinger et al. 2003).

3

Molecular Organization of the Active Zone

Several CAZ-specific constituents have been identified and characterized at the molecular level, including the UNC13/Munc13 proteins, the Rab3-interacting molecules (RIMs), Bassoon, Piccolo/Aczonin and the ERC2/CAST (Garner et al. 2000; Rosenmund et al. 2003; Ziv and Garner 2004). These proteins are physically interconnected and thus thought to form the molecular

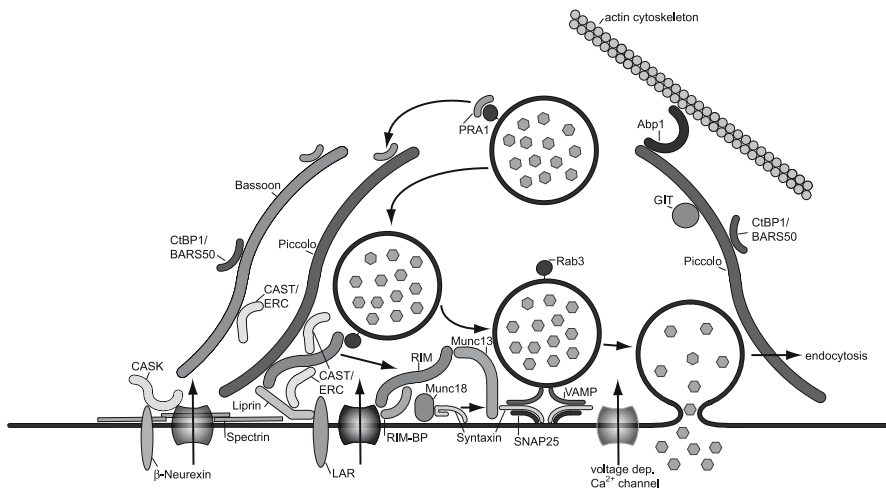


Fig. 1 Molecular organization of the CAZ. The CAZ-specific proteins RIMs, Munc13s, Bassoon, Piccolo and CAST/ERC are thought to localize and organize membrane trafficking events of the synaptic vesicle cycle and connect it to the active zone membrane proteins including voltage-gated Ca^{2+} channels and cell adhesion molecules such as the neuexins. Further components that are not exclusive CAZ components include Ca^{2+} /calmodulin kinase domain-containing membrane-associated guanylate kinase CASK, the transcriptional co-repressor CtBP1/BARS50, the RIM-binding proteins (RIM-BP), the prenylated Rab3 acceptor protein PRA1, the ARF-GTPase-activating protein GIT, the receptor tyrosine phosphatase LAR and its interacting protein Liprin, components of the SNARE complex and its control elements (e.g. Munc18). The interaction between Piccolo and the actin-binding protein Abp1 is thought to link the active zone to the neighboring endocytic zone. For further details see: Gundelfinger, Altmann, Fejtova, Active zone. In: *Encyclopedic Reference of Neuroscience* (Field editor: M. Takahashi, Springer Berlin, in press). The picture was taken from the website of the IfN (http://www.ifn-magdeburg.de/en/departments/neurochemistry_and_molecular_biology)

Table 1 Proposed protein-protein interactions of CAZ proteins

Protein (synonyms)	Domains/motifs	Interaction partners [Refs.]	Proposed function for the interaction
Rab3 interacting molecules (RIMs: primarily RIM1 α ; RIM2 α)	Zn finger	Rab3 [1]	SV tethering?, Regulation of SV exocytosis?
		Munc13-1 [2]	SV priming
		ubMunc13-2 [2]	SV priming
	PDZ	CAST1/ERC2 3,4	Scaffolding
	C2A	Piccolo [5]	Scaffolding
		N-type voltage-dependent Ca ²⁺ channel [6]	Channel anchoring
		Synaptotagmin, SNAP-25 [6]	Ca ²⁺ sensing
	Proline-rich sequence (PRS)	RIM-BPs [7]	Scaffolding
	C2B	α -Liprin [8]	Scaffolding
		N-type voltage-dependent Ca ²⁺ channel [6]	Channel anchoring
Munc13s		Synaptotagmin, SNAP-25 [6]	Ca ²⁺ sensing
	N-term region of Munc13-1 and ubMunc13-2	RIM1 α [2]	Scaffolding, Rab3 effector
		Calmodulin [9]	Ca-dep. plasticity
	Conserved R region of Munc13s	DOC2 α (double C2 domain protein) [10]	Unknown
		Spectrin β -spIII Σ [11]	Cytoskeleton anchoring
		msec7-1 ARF-GEF [12]	Cytoskeleton regulation
		Syntaxin [13]	SV fusion, SNARE complex regulation
		CtBP1/BARS-50 (lysophosphatidic acid acyl transferase, LPAAT) [14]	Membrane trafficking? Regu- lation of membrane curvature?
		Ribeye/CtBP2 (LPAAT) [14]	Membrane trafficking?, Scaffolding
		CAST/ERC [15]	Scaffolding
Bassoon	Zn fingers		
	N-terminal of CC2		
	CC3		

Table 1 (continued)

Protein (synonyms)	Domains/motifs	Interaction partners [Refs.]	Proposed function for the interaction
Piccolo (Aczonin)	Q domain	Actin-binding protein Abp1 [16]	Actin binding, link to endocytosis
	Zn fingers	PRA1 [17]	Unknown
	PRS	GIT (ARF-GAP) [18]	GTPase regulation, membrane trafficking
	PRS	Profilin (actin binding protein) [19]	Actin regulation
	CC3	CAST/ERC [15]	Scaffolding
	PDZ	cAMP-GEFII [5]	GTPase regulation
	C2A	RIM2 [5]	Scaffolding, Rab3 effector
	C2B	Piccolo [5]	Scaffolding
	Coiled-coil regions	L-type voltage-dependent Ca ²⁺ channel [5]	Channel anchoring
		Bassoon, Piccolo [15]	Scaffolding
CAZ-associated structural proteins (CASTs, ERCs)	C-term PDZ binding motif SH3 domains (one of 3) SH3 domains (one of 3)	α -Liprin [20]	Scaffolding, Transport?
		RIMs [3]	Scaffolding
		RIMs [7]	Scaffolding
		Ca ²⁺ channels Ca _v 2.2 (N-type), Ca _v 1.3 (L-type) [21]	Channel anchoring
α -Liprins (SYD-2)	N-term CC region	CAST/ERC [20]	Scaffolding, Transport?
	C-term SAM domains	KIF1A (kinesin motor) [22]	Transport
		LAR (receptor tyrosine phosphatase) [23] GRIP [24]	Receptor anchoring Receptor clustering

Table 1 continued*References to Table 1:*

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scaffold of the CAZ (Fig. 1, Table 1). In addition, functions in organizing the SV cycle have been assigned to some of the CAZ-specific proteins.

3.1

The UNC13/Munc13 Protein Family – Key Actors in SV Priming

UNC13 proteins, first identified in “uncoordinate” mutants of *Caenorhabditis elegans*, contain two or three C2 domains, which are supposed to be involved in Ca^{2+} and phospholipid binding, and one C1 domain that can bind the second messenger DAG and β -phorbol esters (for review, see Brose et al. 2000). Alternative splicing of the *C. elegans unc13* gene gives rise to two isoforms differing in their N-terminal sequence. In vertebrates, four UNC13 genes have been identified encoding Munc13-1, Munc13-2, Munc13-3, and Munc13-4. In analogy to worm UNC13, two isoforms arise from the *Munc13-2* gene: bMunc13-2 is similarly to Munc13-1 and Munc13-3 brain specific and ubMunc13-2 is ubiquitously expressed (Koch et al. 2000). Genetic studies in *C. elegans*, *Drosophila* and mice indicate that UNC13 proteins have an important role in the attainment of fusion competence of docked

SVs, a process generally called SV “priming” (Brose et al. 2000). According to our current view, Munc13s bind the presynaptic membrane SNARE protein syntaxin and promote the formation of a loose SNARE complex, which is a prerequisite for Ca^{2+} -dependent SV fusion (Rosenmund et al. 2003). Several second messengers and regulatory proteins that interact with UNC13 proteins can fine-tune their function. For example, DAG and phorbol esters bind the C1 domain of Munc13s. Electrophysiological studies on mice expressing a DAG-insensitive Munc13-1 variant have demonstrated that DAG binding to Munc13 is essential for the maintenance of efficient transmission during trains of action potentials and that Munc13s, and not protein kinase C, are the targets for DAG and phorbol ester-mediated potentiation of transmitter release (Rhee et al. 2002). Moreover, Munc13s possess an evolutionary conserved Ca^{2+} -dependent calmodulin (CaM) binding motif. The Ca^{2+} sensor-effector complex regulates synaptic vesicle priming and synaptic efficacy by sensing residual Ca^{2+} concentrations; in this way, it controls short-term plasticity characteristics during periods of repeated synaptic activity (Junge et al. 2004).

Rosenmund and colleagues demonstrated a differential contribution of Munc13-1 and Munc13-2 to the regulation of presynaptic short-term plasticity (Rosenmund et al. 2002). While synapses containing Munc13-1 depress during trains of action potentials, synapses containing Munc13-2 augment when repeatedly stimulated. As both isoforms can be localized to distinct presynaptic boutons of a single neuron, the same train of action potentials can lead to divergent responses at these synapses. These differences in regulation of short-term plasticity by Munc13 isoforms vanish when CaM insensitive mutants of Munc13-1 and Munc13-2 are expressed (Junge et al. 2004).

3.2

RIMs – Multidomain Rab3 Effectors with a Major Organizing Role

While invertebrates possess only a single gene for the RIM (Rab3-interacting molecule) homologue UNC10, in vertebrates four RIM-related genes have been identified that code for RIM1 α , RIM2 α , β and γ , RIM3 γ and RIM4 γ isoforms. The RIM2 gene is transcribed from alternative promoters to give rise to α , β and γ isoform. The diversity of RIM family members is even increased by alternative splicing of α and β isoforms (Wang et al. 2000; Wang and Sudhof 2003). The α isoforms comprise an N-terminal zinc finger domain, a PDZ (PSD-95/SAP90-DlgA-ZO-1) domain and two C2 domains, the RIM2 β isoform lacks the zinc finger domain, and γ isoforms include just one C-terminal C2 domain. RIMs are expressed predominantly in brain; both α isoforms are specific constituents of the CAZ.

RIM1 α was first identified as an effector of the monomeric GTPase proteins of the Rab3 subfamily that are associated with SVs (Wang et al. 1997). This suggested a role for RIMs in the regulation of neurotransmitter release. Genetic

studies in *C. elegans* revealed a function of the RIM homologue UNC10 in SV priming, similar to that of UNC13 proteins (Koushika et al. 2001). The functional relationship of the molecules was supported by showing an interaction of zinc finger of RIM1 α , which interacts also with Rab3, with the N-terminal region of Munc13-1 and ubMunc13-2 (Betz et al. 2001). A detailed study of RIM1 α knock-out mice revealed multiple modulatory roles for RIM1 α during synaptic transmission (Schoch et al. 2002). RIM1 α is phosphorylated by protein kinase A (PKA) and its phosphorylation is necessary for binding the adaptor protein 14-3-3. This interaction is crucial for manifestation of mossy fibers LTP that has a principal presynaptic component and is dependent on PKA activation, as shown by the absence of mossy fiber LTP in RIM1 α -deficient mice, by expression of non-phosphorylatable RIM1 or by expression of RIM1 α -binding unstable 14-3-3 mutant (Castillo et al. 2002; Lonart et al. 2003; Simsek-Duran et al. 2004). Schaffer collateral synapses on CA1 pyramidal cells exhibited decreased neurotransmitter-release probability and abnormalities in short-term plasticity in RIM1 mutants. Remarkably, the effect of RIM1 α was inverse in excitatory as compared with inhibitory synapses, suggesting diversity of release controlling protein complexes present at these synapses (Schoch et al. 2002). In contrast to well-understood role of RIM1 α in mossy fiber LTP, the molecular basis of these phenotypes has not yet been established.

The interaction with Munc13s and Rab3 can account only partially for the above-mentioned findings. Additional interaction partners of RIM described recently could play a role here (see Table 1, Fig. 1). For instance, RIMs can associate via their C2 domains with the α 1B (Ca_v2.2) pore-forming subunit of N-type Ca²⁺-channels, but not with the α 1D (Ca_v1.3) subunit of L-type channels. This interaction seems to be independent of the Ca²⁺ concentration and is competitive with the binding of channels to the C2 domain of syntaxin1 (Coppola et al. 2001). The C2 domains of RIM further bind the target membrane SNARE protein SNAP-25 and the C2B domain can oligomerize with the C2 domain of the presynaptic Ca²⁺ sensor synaptotagmin 1 in a Ca²⁺-dependent manner (Coppola et al. 2001; Schoch et al. 2002). RIM1 α and RIM2 α and β contain a conserved SH3-domain binding motif mediating an interaction with the SH3 domain of RIM binding proteins RIM-BP1 and RIM-BP2 that bind via the same domain α 1B (Ca_v2.2) and α 1D (Ca_v1.3) subunits of N- and L-type Ca²⁺ channels, respectively (Wang et al. 2000; Hibino et al. 2002). As RIM-BPs contain three SH3 domains they potentially can bind RIMs and Ca²⁺ channels simultaneously. The above-described interactions could contribute to the changes in short- and long-term plasticity observed in RIM1 α -deficient mice. They link RIMs physically with the Ca²⁺-triggered machinery that executes and fine-tunes the SV exocytosis. Experiments performed in pancreatic β cells revealed an interaction of PDZ domain of RIM2 α with the cAMP-guanidine nucleotide exchange factor II (cAMP-GEFII) that mediates cAMP-dependent PKA-independent exocytosis in pancreatic β -cells. This implicates a role of RIM2 α in regulation of exocytosis also in secretory cells (Ozaki et al. 2000).

The C2B domain of RIMs can bind α -liprins in a Ca^{2+} -independent manner (Schoch et al. 2002). Liprins were originally identified as intracellular binding partners for LAR receptor protein tyrosine phosphatases implicated in axonal guidance, synaptogenesis and morphogenesis of non-neuronal tissues (Serra-Pages et al. 1998; Dunah et al. 2005). In this way, liprins are responsible for the appropriate localization and clustering of LAR. Studies on *C. elegans* *syd-2* (*liprin*) mutants have shown liprin to be required for proper assembly of the active zone (Zhen and Jin 1999) and that RIM is mis-localized in *syd-2* mutants (discussed in Schoch et al. 2002). The interaction of RIMs with liprins can play a role in formation and organization of molecular scaffold at the active zone.

RIMs interact also with CAST/ERC protein family members and with Piccolo (Ohtsuka et al. 2002; Wang et al. 2002; Shibasaki et al. 2003) (see Fig. 1, Table 1 for details).

3.3

CAST/ERC Proteins – Major Structural Proteins of the CAZ?

The CAST/ERC (CAZ-associated structural protein/ELKS-Rab6-interacting protein-CAST) protein family is conserved from worms to mammals, where it is represented by two genes: CAST1/ERC2 and ERC1. The latter is expressed in two alternatively spliced isoforms diverging in their C-terminus, i.e. brain-specific ERC1b and ubiquitously expressed ERC1a (Ohtsuka et al. 2002; Wang et al. 2002; Deguchi-Tawarada et al. 2004). CAST/ERC2 and ERC1b are localized at the active zone. Their C-termini resemble canonical PDZ recognition motifs that mediate interaction with the PDZ domain of RIMs. This interaction seems to be essential for targeting RIMs to the active zone during synaptogenesis (Wang et al. 2000; Ohtsuka et al. 2002). CAST/ERC proteins have three coiled-coiled domains. The second coiled-coiled domain of CAST/ERC2 can interact with Piccolo or Bassoon, thereby forming a tetrameric complex composed of CAST/ERC2, Bassoon or Piccolo, RIM1 α and RIM's binding partner Munc-13 (Takao-Rikitsu et al. 2004). Disruption of CAST/ERC binding to Bassoon inhibits neurotransmitter release by not yet understood mechanisms. Furthermore, CAST/ERCs can interact with α -liprins (Ko et al. 2003). Over-expression of N-terminal, the portion of CAST bearing the interaction domain, enhances synaptic accumulation of α -liprins. Thus, this interaction could contribute to the molecular organization of active zone.

3.4

Piccolo and Bassoon – Two Related Giants Scaffolding the CAZ

Piccolo/Aczonin (Cases-Langhoff et al. 1996; Wang et al. 1999; Fenster et al. 2000) and Bassoon (tom Dieck et al. 1998) are giant proteins of 530 kDa and

420 kDa, respectively, sharing significant sequence similarity. They are evolutionarily conserved in vertebrates, but have no identifiable homologues in worms or flies. Bassoon and probably also Piccolo can be post-translationally modified by *N*-myristoylation (Dresbach et al. 2003). They contain 10 regions of particular sequence similarity called Piccolo-Bassoon homology (PBH) domains. Three of the PBH domains include coiled-coil domains. Embedded within the first two PBH domains are two zinc fingers that exhibit limited homology to the zinc fingers of RIM α -isoforms. The zinc fingers of Piccolo have been found to bind the prenylated Rab acceptor (PRA1) (Fenster et al. 2000), a small protein that can interact with both VAMP2/synaptobrevin 2 as well as with Rab3. This may indicate a link to the exocytic apparatus for SVs (Martincic et al. 1997). The *N*-terminal proline-rich Q domain of Piccolo, which is absent from Bassoon, interacts with the F-actin-binding protein Abp1 and heterologous over-expression of this domain interferes with clathrin-mediated endocytosis (Fenster et al. 2003). Moreover, Piccolo, but not Bassoon, also interacts with GIT proteins that function as signaling integrators in processes of membrane trafficking, reorganization of the actin cytoskeleton and endocytosis (Kim et al. 2003). Both Bassoon and Piccolo interact with both members of the CtBP (C-terminal binding protein/Connected to Bassoon and Piccolo) protein family (tom Dieck et al. 2005). CtBPs were initially described as nuclear regulator of gene expression interacting with numerous transcription factors (Furusawa et al. 1999). In retinal cells, CtBP2 is transcribed from a specific promoter giving rise to protein Ribeye, which is a core component of photoreceptor ribbons (Schmitz et al. 2000). CtBP1 acts in control of gene expression, but was also localized at the Golgi complex, where it regulates vesicle fission from the trans-Golgi network and is a substrate for brefeldin A-ADP-ribosylation, hence its synonymous name BARS50 (brefeldin A-ADP-ribosylation substrate of 50 kDa) (Schaeper et al. 1995; Weigert et al. 1999). Recently, CtBP1/BARS50 was described also as neuronal synaptic protein (tom Dieck et al. 2005). Additional common interaction partners of Bassoon and Piccolo are members of the CAST/ERC protein family (see above).

A unique PDZ and C2A and C2B domains structurally related to those of RIMs are located in the C-terminal region of Piccolo. This C-terminal region of Piccolo has no counterpart in Bassoon. The C2A domain of Piccolo has unique Ca^{2+} -binding properties. It binds Ca^{2+} with high specificity but low affinity, and the binding causes major conformational changes of overall domain. Moreover, Ca^{2+} binding is a prerequisite of interaction of C2A domain with many interaction partners of Piccolo (see below). Piccolo could therefore function as a Ca^{2+} sensor in processes of short-term presynaptic plasticity where Ca^{2+} levels rise after repeated stimuli (Gerber et al. 2001). Piccolo is expressed in many alternatively spliced variants (Fenster and Garner 2002; Garcia et al. 2004). Alternative splicing of nine residues within the C2A domain has an enormous effect on its Ca^{2+} binding properties; it

increases its Ca^{2+} affinity, abrogates Ca^{2+} -dependent dimerization and abolishes the Ca^{2+} -induced conformational change, resulting in characteristics of typical C2 domains (Garcia et al. 2004). The C2B domain that can be alternatively spliced out lacks the four of five aspartate residues usually crucial for Ca^{2+} binding in C2 domains. It also has other abnormalities of tertiary structure and therefore it is probably incapable of Ca^{2+} binding (Gerber et al. 2001).

Similar to large RIM isoforms, Piccolo can interact with cAMP-GEFII via its PDZ domain, as has been shown for pancreatic β -cells (Fujimoto et al. 2002). Treatment of β -cells with antisense oligonucleotides against Piccolo results in reduced cAMP-induced PKA-independent secretion of insulin. The C2A domain of Piccolo can form homodimers as well as heterodimers with RIM2 in a Ca^{2+} -dependent manner. Moreover, both C2 domains of Piccolo have been shown to interact with the $\alpha 1\text{C}$ ($\text{Ca}_v1.2$), but not with $\alpha 1\text{D}$ ($\text{Ca}_v1.3$) subunit of *L*-type voltage-dependent Ca^{2+} channels that regulates Ca^{2+} influx into pancreatic β -cells (Shibasaki et al. 2003). These findings suggest that Piccolo could together with RIM2s contribute to the organization of a special microdomain in pancreatic β -cells that is involved in secretion, and thereby function as Ca^{2+} sensor during cAMP-induced PKA-independent exocytosis of insulin granules. Whether the above-described functional interactions of Piccolo can also play a role during neurotransmitter release in neurons remains to be clarified.

Recent studies of mice lacking functional Bassoon provided some insight into the synaptic function of this protein (Altrock et al. 2003; Dick et al. 2003). These mice express a residual 180-kDa Bassoon protein, which lacks the central region involved in proper anchoring of the protein to the CAZ (Altrock et al. 2003; Dresbach et al. 2003). Studies on autapse-forming microisland cultures and hippocampal slices from *Bsn* mutants suggest that a significant fraction of hippocampal glutamatergic synapses is functionally inactive, but can morphologically not be distinguished from functional synapses. This phenotype is partly reminiscent of the phenotype of Munc13-1 mutant mice and suggests a function of Bassoon in priming of SVs by yet unknown molecular mechanisms.

4

Synaptic Ribbons – Specializations of the CAZ at High-Throughput Synapses

Ribbon synapses are highly specialized synapses adapted to tonic or high throughput neurotransmitter release that requires high precision and reliability. These synapses are characterized by electron-dense structures associated with the active zone membrane – the ribbons, which are thought to represent specializations of the CAZ (Garner et al. 2000; Zhai and Bellen 2004). Ribbon synapses are found photoreceptor cells and bipolar cells of

the retina as well as in hair cells of the inner ear (for review, see Fuchs et al. 2003; Sterling and Matthews 2005). A current model suggests that synaptic ribbons serve to tether SVs and to convey them efficiently to the active zone (von Gersdorff 2001). Alternatively, ribbons were proposed as an anchoring device for SV that mediates release of transmitter from multiple vesicles by compound fusion. Basically all CAZ-specific proteins including RIM1 (Wang et al. 1997), Bassoon (Brandstatter et al. 1999), Piccolo (Dick et al. 2001), CAST/ERC2 (tom Dieck et al. 2005) and Munc13-1 (tom Dieck et al. 2005) have also been identified as components of synaptic ribbons of photoreceptor cells. In addition, Ribeye, an isoform of the transcription co-repressor CtBP2 (see above), has been identified as a specific structural component of retinal photoreceptor ribbons (Schmitz et al. 2000).

Bsn mutant mice, which lack the central part of the protein (Altrock et al. 2003), display a very distinctive synaptic ribbon phenotype. In retinal photoreceptors, synaptic ribbons are not associated with the active zone but float freely in the presynaptic terminals, suggesting that Bassoon is involved in anchoring of ribbons to the presynaptic membrane (Dick et al. 2003). As a consequence synaptic transmission from photoreceptors is impaired. A similar observation has been made for ribbons of cochlear inner hair cells (Khimich et al. 2005). Here, a detailed electrophysiological analysis revealed a reduction of the readily releasable SV pool and impaired synchronous auditory signaling.

To gain a more detailed insight into the molecular organization of synaptic ribbons in particular, and the CAZ in general, the association of CAZ components with floating ribbons and the presynaptic membrane was assessed in *Bsn* mutants and compared with wild-type mice. While Piccolo, Ribeye, CtBP1, RIM1 and the ribbon-associated motorprotein KIF3a were found in floating ribbons, RIM2, Munc13-1 and CAST were associated with the active zone as defined by the presence of voltage-gated Ca^{2+} channels (tom Dieck et al. 2005). Bassoon was exclusively localized at the interface between the ribbon and the active zone associated arciform density in wild-type ribbon synapses (Brandstatter et al. 1999; tom Dieck et al. 2005). As the binding site for Ribeye is lacking in the mutant Bassoon protein, it was suggested that ribbon anchoring may depend on physical interaction between Bassoon and Ribeye. Bassoon's binding partner on the other, i.e. active zonal, side may be CAST/ERC2, which has also been shown to interact with Bassoon (Takao-Rikitsu et al. 2004 see above). The differential distribution of Bassoon and Piccolo along synaptic ribbons may explain why Piccolo cannot compensate for the lack of functional Bassoon in the mutant photoreceptor cell. Detailed analyses of the exact distribution and localization of CAZ components at conventional synapses by EM immunogold studies will have to unravel, whether a similar molecular organization forms the scaffold of the CAZ at conventional brain synapses.

5

Developmental Assembly of the Active Zone – The Active Zone Precursor Vesicle Hypothesis

During development, a previously undetermined region of axonal plasma membrane differentiates into a presynaptic active zone with a highly complex and organized molecular composition that warrants neurotransmitter release with high specificity and fidelity in precise alignment with the postsynaptic receptive apparatus.

Our understanding of the molecular and cellular mechanism underlying this process is surprisingly vague compared with our knowledge about the presynaptic function. Synapse formation is initiated by axonal growth cones or highly mobile dendritic filopodia that grow out and sense their environment until they reach a suitable counterpart (reviewed by Vaughn 1989). New axo-dendritic contacts are stabilized by adhesion molecules (reviewed by Scheiffele 2003). The activity-induced SV recycling can be observed within 30 min after the axo-dendritic contact formation and the active-zone protein Bassoon is recruited to these sites already within 10 min (Ahmari et al. 2000; Friedman et al. 2000). The amazing velocity of this process is reasonable assuming that the synapse formation and remodeling underlies the process of learning and memory formation, but is difficult to imagine from a molecular perspective. The hypothesis, initially brought up by Vaughn in the late 1980s, that propose the formation of presynaptic specializations from preassembled molecular complexes associated with vesicular structures, became attractive in this context (Vaughn 1989). This hypothesis was largely supported by electron microscopy (EM) studies demonstrating presence of different pleomorphic vesicular structures, dense-core vesicles, sometimes with spicules projecting from their surface, and coated vesicles in nascent synapses (Ahmari et al. 2000; Zhai et al. 2001). Similarly, live imaging studies indicated presence of mobile clusters labeled with a green fluorescent protein (GFP)-tagged SV molecule synaptobrevin/VAMP2 (Ahmari et al. 2000) or with a GFP-tagged active zone protein Bassoon (Zhai et al. 2001; Shapira et al. 2003) traveling within axons of young cultured hippocampal neurons and their rapid accumulation at the sites of new axo-dendritic contacts. The electron-microscopic, immunocytochemical and biochemical analysis of these clusters revealed two distinct vesicular structures: first, clear vesicles of 50–60 nm bearing SV specific proteins like VAMP2/synaptobrevin 2, synapsin, synaptotagmin and second, a previously unknown, dense-core vesicles of 80 nm containing active zone specific proteins such as Piccolo, Bassoon and CAST/ERC that were termed Piccolo-Bassoon transport vesicles (PTVs) (Vaughn 1989; Ahmari et al. 2000; Zhai et al. 2001; Ohtsuka et al. 2002). These two vesicle populations show similar mobility characteristics, but they seem not to form part of the same mobile cluster. PTVs are not stained with synapsin I, synaptophysin or synaptobrevin antibody (Zhai et al. 2001; Shapira et al. 2003) in contrast to the mobile SV

precursors described by Ahmari and co-workers (Ahmari et al. 2000). Besides Bassoon, Piccolo and its interaction partners CASTs/ERCs and CtBP-1, PTVs carry also RIM, Munc13, Munc18, syntaxin, SNAP-25, Rab3 and $\alpha 1$ and $\beta 1$ subunits of N -type Ca^{2+} channel implied in SV exocytosis and cell-adhesion molecules like N -cadherin (Zhai et al. 2001; Ohtsuka et al. 2002; Shapira et al. 2003; and our unpublished observations). Several proteins such as syntaxin, SNAP-25, Rab3 or N -cadherin were found to be associated with both PTVs and SV precursors. PTVs were proposed to represent the active zone precursor vesicles, capable to fuse with the presynaptic plasma membrane and thereby constitute a new active zone (Fig. 2). Quantitative immunocytochemical and live imaging studies confirmed that presynaptic sites could rapidly be assembled in unitary fashion from PTVs and that recruitment of 2–5 PTVs precedes regulated SV recycling (Shapira et al. 2003; Bresler et al. 2004). The active zone components delivered by PTV are believed to provide a scaffold for recruiting of SV possibly by trapping of preformed SV clusters (Ahmari et al. 2000; Friedman et al. 2000). The mechanism by which the PTVs are targeted towards and fuse at the place of the new axo-dendritic contacts is currently not known, but it is reasonable to assume that they are targeted by an interaction of PTV-associated molecules with a protein complex organized by the cytoplasmic domains of cell-adhesion molecules such as neurexin, NCAM or

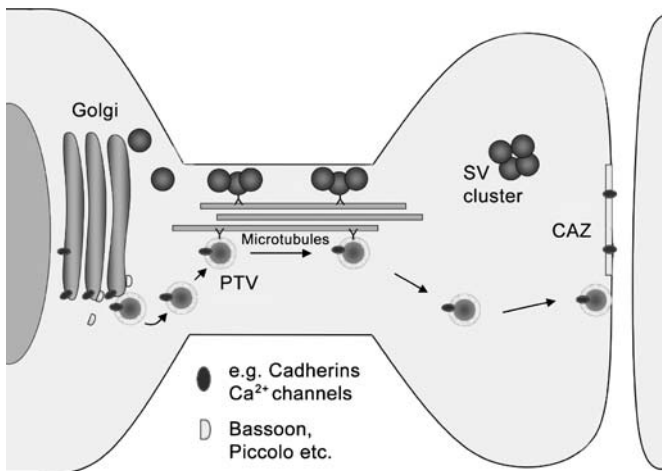


Fig. 2 The active zone transport vesicle hypothesis: PTVs bud from the trans-Golgi-complex and travel along the axon via microtubule-based transport and are directed to nascent active zones by unknown signaling mechanisms. Fusion of PTVs with the axonal plasma membrane results in formation of an active zone. The topology of membrane fusion predicts that the CAZ proteins traveling on PTVs become located immediately underneath the plasma membrane, their contents are released, and transmembrane proteins become incorporated into the plasma membrane. Synaptic vesicle precursors are also generated at the Golgi-complex (*left*) and move along the axon along microtubules using independent pathways (adapted from Dresbach, Altrock, Gundelfinger, Neuroforum 03/2003)

SynCAM that are present at newly formed axo-dendritic contacts. However, the nature of these interactions is currently completely obscure and remains to be characterized.

Several recent studies have focused on synaptic targeting of various proteins associated with PTVs. Munc13 seems to be recruited to synapses by its interaction partner RIM (Schoch et al. 2002). However, other RIM interacting molecules are recruited in a RIM independent way (Schoch et al. 2002). RIM itself requires for its synaptic localization the PDZ domain that mediates the interaction with CAST/ERC2 (Ohtsuka et al. 2002; Wang et al. 2002). ERC2 was identified to regulate the targeting of its interaction partner α -liprin (Ko et al. 2003). Moreover, ERC2 interacts with Bassoon and Piccolo, but disruption of the ERC2-interaction domain of Bassoon or the Bassoon-interacting domain of ERC2 did not lead to their mis-localization (Takao-Rikitsu et al. 2004). A targeting study of various Bassoon deletion constructs revealed that both its myristoylated *N*-terminal region and its central region could be independently targeted to the active zone (Dresbach et al. 2003). However, these studies did not rule out the possibility of oligomerization of Bassoon and ERC2 deletion mutants with endogenous wild-type proteins. EM and immunocytochemical studies as well as imaging of GFP-tagged Bassoon in neurons suggest that PTVs are assembled at the Golgi apparatus within the cell body and transported along the microtubuli towards the axonal endings (Dresbach et al., 2006).

In conclusion, various studies during recent years have confirmed that presynaptic specializations are formed at the site of new axo-dendritic contacts by incorporating preassembled molecular complexes that are built up in the cell body and transported associated with vesicular organelles along the microtubules into axons toward presynaptic sites of synaptogenesis. PTVs carrying preassembled components of active zone have been described in young neurons, but they are sparse in differentiated neurons. Whether the turnover of active zone components and synaptogenesis in adult CNS rely on alternative transport and assembly mechanisms is an urgent question that remains to be solved.

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Extracellular Matrix and Synaptic Functions

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Abstract Comprehensive analysis of neuromuscular junction formation and recent data on synaptogenesis and long-term potentiation in the central nervous system revealed a number of extracellular matrix (ECM) molecules regulating different aspects of synaptic differentiation and function. The emerging mechanisms comprise interactions of ECM components with their cell surface receptors coupled to tyrosine kinase activities (agrin, integrin ligands, and reelin) and interactions with ion channels and transmitter receptors (Narp, tenascin-R and tenascin-C). These interactions may shape synaptic transmission and plasticity of excitatory synapses either via regulation of Ca^{2+} entry and postsynaptic expression of transmitter receptors or via control of GABAergic inhibition. The ECM molecules, derived from both neurons and glial cells and secreted into the extracellular space in an activity-dependent manner, may also shape synaptic plasticity through setting diffusion constraints for neurotransmitters, trophic factors and ions.

Abbreviations

AChR	acetylcholine receptor
AMPA	alpha-amino-3-hydroxy-5-methyl-4 isoxazolepropionic acid
ApoER2	apolipoprotein E receptor 2
CA1, 3	hippocampal <i>cornu ammonis</i> regions 1,3
CaMKII	Ca^{2+} /calmodulin-dependent protein kinase II
CNS	central nervous system
CREB	cAMP-responsive element-binding protein
CS	chondroitin sulfate
CSPG	chondroitin sulfate proteoglycan
ECM	extracellular matrix
EPSC	excitatory postsynaptic current
FN	fibronectin
GABA	gamma-aminobutyric acid
GIRK	G-protein-coupled inwardly rectifying K^{+} channel
GluR1	glutamate receptor subunit 1
HA	hyaluronic acid
HB-GAM	heparin-binding growth-associated molecule
HNK-1	human natural killer cell antigen 1
HSPG	heparan sulfate proteoglycan

IAP	integrin-associated protein
KO	knock out
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
MMP	matrix metalloprotease
MuSK	muscle-specific receptor tyrosine kinase
Narp/NP2	neuronal activity-regulated pentraxin
NMDA	<i>N</i> -methyl- <i>D</i> -aspartate
NMJ	neuromuscular junction
NP1	neuronal pentraxin 1
NR2B	NMDA receptor 2B
OPC	oligodendrocyte progenitor cells
PKC	protein kinase C
PSA-NCAM	polysialylated neural cell adhesion molecule
PSI	phosphacan short isoform
RAP	receptor-associated protein
RPTP	receptor protein tyrosine phosphatase
SAP90/PSD-95	synapse-associated protein 90 kDa/post-synaptic density protein of 95 kD
SV2	synaptic vesicle protein 2
TN-C	tenascin-C
TN-R	tenascin-R
TSP	thrombospondin
VDCC	voltage-dependent calcium channel
VLDL	very low density lipoprotein

1

Introduction

The architecture of a tissue is determined by recognition mechanisms that involve not only cell-cell interactions, but also interactions between cells and the extracellular matrix (ECM). In animals, organized groups of cells are surrounded by a matrix formed by collagens, proteoglycans and glycoproteins. Molecules in the matrix not only interact with each other, but also can activate signal transduction pathways via diverse cell-surface receptors. These pathways orchestrate the inputs from equally diverse ECM molecules to coordinate cell functions such as proliferation, migration, and morphological and biochemical differentiation.

In the nervous system, ECM molecules also coordinate synaptogenesis and synaptic functions. The importance of ECM in the synaptic organization is probably most spectacularly highlighted by the structure of the neuromuscular junction (NMJ). An important feature of the NMJ is the prominent basal lamina of ECM that runs through the synaptic cleft. This matrix contains acetylcholine esterase, which degrades the transmitter and thus terminates the presynaptic signal, and houses many of the signaling and structural molecules that are now known to be essential players in the development

and stability of the NMJ. Although a large proportion of extracellular space in the brain is devoid of massive accumulations of ECM components, the extrasynaptic space contains distinct aggregates of ECM molecules, in particular around synapses. Conspicuous structures that are enriched in ECM molecules in the central nervous system (CNS) are the so-called perineuronal nets, which surround cell bodies and proximal dendrites in a mesh-like structure that interdigitates with synaptic contacts. Perineuronal nets are heterogeneous in structure and composition, and nets that are associated with different sets of neurons are characterized by unique molecular compositions. Apart from these spectacular ECM structures, immunoreactivity for many ECM molecules appears as diffuse staining, documenting the widespread occurrence of these molecules in the extrasynaptic space, the structural and molecular organization of which remains to be elucidated.

Over the last decades, substantial knowledge has accumulated with regard to the morphogenetic effects of ECM molecules during development, traumatic lesion and regeneration in the nervous system. In addition, the ECM and associated molecules are involved in physiological processes in the adult brain, such as synaptogenesis and synaptic plasticity, which will be the focus of this review.

2

Synaptogenic Activity of Agrin

The nerve-derived alternatively spliced α isoform of the heparan sulfate proteoglycan (HSPG) agrin proved to be necessary for postsynaptic differentiation of the neuromuscular junction (for a review see Sanes and Lichtman 2001). Mice lacking only the α -exons with normal expression of all other agrin isoforms have a marked reduction in acetylcholine receptor (AChR) clusters and postsynaptic differentiation, and an overgrowth of motor neuron terminals well beyond their normal domain in the central end-plate band of the muscle (Burgess et al. 1999). Activity of agrin involves signaling via the transmembrane muscle specific receptor tyrosine kinase MuSK. Mice that lack MuSK show a phenotype similar to that of agrin knockout (KO) mice, with a complete lack of AChR clustering and presynaptic abnormalities (DeChiara et al. 1996). In addition, muscle fibers cultured from MuSK KO mice do not respond to agrin, and agrin is capable of activating MuSK in a variety of heterologous in vitro systems. A crucial effector downstream of tyrosine phosphorylation activity mediated by MuSK is the cytoplasmic scaffolding protein rapsyn that serves to scaffold together AChRs in the plasma membrane and anchor them to the actin cytoskeleton (Phillips et al. 1991). Ablation of the rapsyn gene in mice also results in a complete failure of AChR clustering, although the receptors still appear to be expressed, albeit diffusely, in the central portion of the muscle and the ex-

tent of the presynaptic overgrowth is less severe in rapsyn KO than in agrin and MuSK KO mice.

Recent findings indicate that agrin is important not only for the formation of NMJs but also for the emergence of synapses between cholinergic preganglionic axons and sympathetic neurons in the superior cervical ganglion (Gingras et al. 2002) and for splanchnic nerve-chromaffin cell cholinergic synapses in rat adrenal gland slices (Martin et al. 2005). In the latter, agrin decreased gap junction-mediated electrical coupling that precedes an increase in nicotinic synaptic transmission. This developmental switch from predominantly electrical to chemical communication is fully operational within one hour and depends on the activation of Src family-related tyrosine kinases.

In hippocampal cultures, the density of presynaptic boutons and vesicular turnover were reduced when agrin expression or function was suppressed by antisense oligonucleotides and specific antibodies (Ferreira 1999; Bose et al. 2000). However, synaptogenesis occurs normally in primary hippocampal and cortical neurons derived from agrin-deficient mice (Li et al. 1999; Serpin-skaya et al. 1999), suggesting the existence of functional redundant mechanisms for agrin and the activation of these compensatory mechanisms in agrin deficient cultures during development. Application of recombinant agrin to cultured cortical neurons induces multiple events that involve tyrosine kinase activation and results in modulation of intracellular Ca^{2+} levels and activity of MAPK and CaMKII, phosphorylation of CREB and induction of expression of the immediate early gene *c-fos* (Hilgenberg et al. 1999; Hilgenberg and Smith 2004). In line with these findings, agrin KO mice show reduced *c-fos* expression and resistance to excitotoxicity and seizures (Hilgenberg et al. 2002). In summary, in several types of synaptic connections, particularly in cholinergic synapses, agrin stimulates activities of tyrosine kinases that affect important aspects of synaptic organization and signaling.

3

Synaptic Functions of Laminins

Laminins are trimeric molecules consisting of an alpha chain, a beta chain, and a gamma chain, arranged in a cross-like structure with their C-termini intercoiled. Mammals express at least five alpha chains, three beta chains and three gamma chains. Distinct genes encode each subunit, and the expression patterns are often dynamic during development. The laminins are known to act as ligands for integrins and other cell-surface receptors such as dystroglycan. In the NMJ laminins are dynamically and precisely localized on muscle fibres, and even within the synaptic cleft.

At the presynaptic site of the NMJ, $\beta 2$ chain-containing laminin binds directly to the N-type calcium channels and induces clustering of chan-

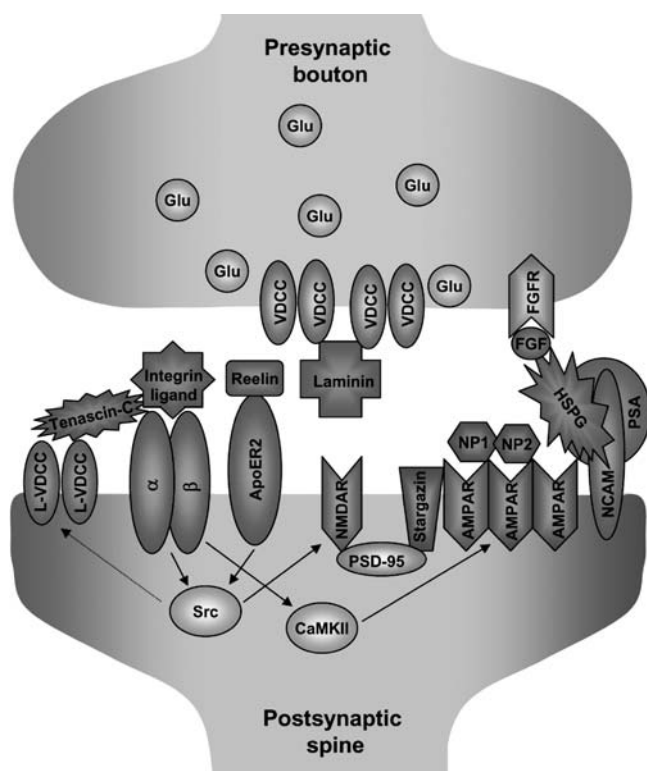


Fig. 1 A summary of mechanisms, by which ECM molecules may shape synaptogenesis and synaptic plasticity. Neuronal pentraxins NP1 and NP2 interact with each other and form a scaffold in the extracellular space, thus promoting clustering of postsynaptic AMPA subtype of glutamate (Glu) receptors (AMPARs) and associated proteins, such as Stargazin, PSD-95 and NMDA receptors (NMDARs). Scaffolds containing laminins cluster presynaptic voltage-dependent Ca^{2+} channels (VDCCs). Similarly, heparan sulfate proteoglycans (HSPGs) in association with PSA-NCAM serve to accumulate FGF and amplify signaling via FGF receptors. Heparan sulfates and PSA also potentiate AMPA receptors. Reelin and integrin ligands may bind to their cell surface receptors (ApoER2 and $\alpha\beta$ integrins) to trigger intracellular cascades resulting in activation of tyrosine kinases of the Src family and tyrosine phosphorylation of effectors, including the NMDA receptor and the L-type VDCC (L-VDCC). Additionally, integrin ligands may activate Ca^{2+} /calmodulin dependent kinase II that regulates the number and activity of AMPA receptors. Tenascin-C affects L-VDCC via an unknown mechanism that may involve a direct interaction with channels or signaling via integrins

nels, which in turn recruits other presynaptic components (Fig. 1). Surprisingly, recruitment occurs independently of Ca^{2+} influx through the channels (Nishimune et al. 2004). Perturbation of this interaction results in the disassembly of neurotransmitter release sites, the so-called active zones, resembling defects previously observed in an autoimmune neuromuscular disorder

Lambert–Eaton myasthenic syndrome. These abnormalities correlate with physiological defects in quantal release and use-dependent modulation of synaptic transmission at the NMJ (Knight et al. 2003). In mice lacking another laminin, $\alpha 4$, active zones and junctional folds of the NMJ form in normal numbers, but not precisely apposed to each other (Patton et al. 2001), suggesting that laminins may be important players in the alignment of pre- and postsynaptic machineries.

Additional data shows a link between vesicle recycling and laminin that is found in synaptosomes in complex with SV2, a synaptic vesicle transmembrane proteoglycan. SV2 binds with high affinity to purified laminin-1, indicating that a synaptic vesicle component might act as a laminin receptor on the presynaptic plasma membrane. These data imply that there may be a link between laminin-dependent regulation of adhesion and the number of unbound SV2 molecules that could recycle at synapses (Son et al. 2000).

In the CNS, data on synaptogenic function of laminins are scarce. Most striking is a report on abnormal rod photoreceptor synapses in laminin $\beta 2$ chain-deficient mice (Libby et al. 1999). In the normal retina, rod photoreceptors form synaptic triads with three postsynaptic elements that invaginate into the base of the photoreceptor in a way that two horizontal cell dendrites are positioned laterally and one bipolar cell dendrite lies centrally. In contrast, in $\beta 2$ chain-deficient animals, another type of synaptic configuration, dyads, with only one or two horizontal cell processes apposed to the presynaptic specialization (ribbon), are most common. In these mice, “floating synapses”, where a fully formed ribbon without any postsynaptic element apposed, are observed unusually often.

This may be just the tip of the iceberg, since messenger RNAs for all laminin chains could be detected in the CNS, e.g. in the hippocampus. Immunohistochemical analysis showed that $\alpha 5$, $\beta 1$ and $\gamma 1$ are most highly expressed in neuronal cell layers. Thus, laminin-10 ($\alpha 5$ - $\beta 1$ - $\gamma 1$) may be a major isoform in the mouse hippocampus. Interestingly, laminins including laminin-10 are degraded by the tissue plasminogen activator/plasmin protease system (Indyk et al. 2003). Preincubation of cultured hippocampal slices with plasmin inhibited stabilization of long-term potentiation (LTP) at hippocampal CA1 synapses, whereas preincubation with laminin antibodies prevented both the degradation of laminins and the impairment of LTP by plasmin (Nakagami et al. 2000). These data support a view that laminin-mediated interactions between cells and the ECM might be necessary for the maintenance of LTP. A recent genetic study demonstrates that a defect in $\alpha 2$ -containing laminins causes a disruption in long-term synaptic plasticity at the Purkinje cell-parallel fibre synapse in the cerebellum (Anderson et al. 2005). Additional support for the role of laminins in LTP is based on extensive data showing that integrins as the main laminin receptors are involved in synaptic plasticity and synaptogenesis.

4

Synaptic Functions of Integrins

Integrins are transmembrane heterodimeric receptors that are composed of α and β subunits. There are 16 different α and eight different β mammalian integrin subunits, which associate to form 22 recognized $\alpha\beta$ heterodimers. Each integrin recognizes specific ligands, often extracellular matrix molecules such as laminin and fibronectin (FN), or cell adhesion molecules. Many distinct integrin heterodimers are expressed in the developing and adult hippocampus, as detected by in situ hybridization and RT-PCR (Pinkstaff et al. 1999; Chan et al. 2003). Three integrin subunits, $\alpha 8$, $\beta 8$, and $\beta 1$, have been localized by electron microscopic studies to dendritic spines of pyramidal neurons or granule cells in the hippocampus, where they are associated with the post-synaptic densities (Einheber et al. 1996; Nishimura et al. 1998; Schuster et al. 2001). At the light microscopic level, the $\alpha 5$ and $\alpha 3$ subunits were found to be expressed on dendrites of pyramidal cells of the hippocampus and the neocortex (Bi et al. 2001; Chan et al. 2003).

Initial evidence for a potential role of integrins in synaptic plasticity has been gathered by attenuating the stability of hippocampal LTP using broad-spectrum peptide ligands of the integrins (Staubli et al. 1990, 1998). Injection of snake toxins (disintegrins), which preferentially inhibit the binding of ligands to $\beta 1$ - or $\beta 3$ -containing integrins, was very efficient in blocking the stabilization of LTP (Chun et al. 2001), whereas the blocking antibody to $\alpha 3$ subunit facilitated depotentiation, i.e. reversal of LTP by low-frequency stimulation (Kramar et al. 2002). Since laminin, an important ECM component in the nervous system, modulates stabilization of LTP and the $\alpha 3$ subunit was found to be associated with the $\beta 1$ subunit in synaptosomal preparations (Chan et al., 2003), it is plausible that interactions between $\alpha 3\beta 1$ integrin receptors and laminins are important for stabilization of LTP.

Another heterodimer, $\alpha V\beta 3$ integrin, turned out to be necessary for maturation of excitatory hippocampal synapses, converting immature hippocampal synaptic contacts, which express the NR2B subunit of NMDA receptors and have a high glutamate release probability, into mature synapses, which lack NR2B and have a lower release probability (Chavis and Westbrook 2001). Since RGD peptides and $\beta 1$ antibodies interfere with the formation of synapses in organotypic slice cultures, $\beta 1$ -containing integrins may also play a role in synaptogenesis (Nikonenko et al. 2003b). A recent study suggests that interaction between astrocytes and neurons may elicit protein kinase C (PKC) activation via integrin receptors. This activation, initially focal, soon spread throughout the entire neuron and facilitates synaptogenesis throughout the neuron (Hama et al. 2004).

The roles of integrins in synaptic plasticity and learning are also supported by genetic studies. In *Drosophila*, the disruption of *Volado*, a gene encoding two forms of α -integrin, impairs olfactory learning (Grotewiel et al. 1998).

Disruption of the integrin-associated protein IAP produces memory deficits in mice (Chang et al. 1999). Heterozygous mice with genetically reduced expression of the $\alpha 3$ subunit fail to maintain CA1 LTP. Mice with reduced expression of the $\alpha 3$ and $\alpha 5$ subunits also exhibit a defect in paired-pulse facilitation. Mice with reduced expression of $\alpha 3$, $\alpha 5$, and $\alpha 8$ subunits are defective in CA1 LTP and spatial memory in the water maze but have normal fear conditioning (Chan et al. 2003). These results demonstrate that multiple integrins are involved in synaptic plasticity and provide the evidence of their requirement for learning and memory in vertebrates.

Several recent studies highlight potential mechanisms by which integrins may affect synaptic functions. Infusion of a broad spectrum integrin ligand (the peptide GRGDSP) into rat hippocampal slices reversibly increased the slope and amplitude of excitatory postsynaptic potentials mediated by AMPA receptors (Kramar et al. 2003). Biochemical analysis directly demonstrated that the peptide and the native ligand FN trigger $\beta 1$ -dependent rapid and robust increases in tyrosine phosphorylation of focal adhesion kinase, proline-rich tyrosine kinase 2 and Src family kinases (Bernard-Trifilo et al. 2005). In agreement with these data, the ligand-induced increase in AMPA receptor-mediated currents was blocked by inhibitors of the Src tyrosine kinases (Kramar et al. 2003). Src activity was also found to underlie tyrosine phosphorylation of NMDA receptor subunits NR2A and NR2B and a strong increase in NMDA receptor-mediated currents (Fig. 1) (Lin et al. 2003; Bernard-Trifilo et al. 2005). The same signaling pathway may be involved in the regulation of activity of L-type Ca^{2+} channels (Davis et al. 2001; Gall et al. 2003), also playing an important role in induction of some forms of synaptic plasticity.

In summary, available data suggest that at least $\alpha V\beta 3$, $\alpha 3\beta 1$, $\alpha 5\beta 1$ integrins are involved in regulation of synaptic functions. It is likely that activity-dependent release of ECM components or their partial proteolytic cleavage—resulting in relief of cryptic integrin-binding sites—may trigger integrin signaling during LTP, which involves activation of Src tyrosine kinase and modulation of NMDA receptors and the L-type Ca^{2+} channels.

5

Thrombospondins and Synaptogenesis

The thrombospondins (TSPs) constitute a gene family with five members in vertebrates. All TSPs are extracellular multimeric, multidomain calcium-binding glycoproteins that function at cell surfaces and in the extracellular matrix (Adams 2001). Their well-known functions are to promote cell attachment and to regulate the cytoskeleton, migration, and angiogenesis. In the nervous system, four TSPs are expressed: TSP1 and TSP2 by astrocytes during postnatal development, TSP3 predominantly in embryonic brains, and

TSP4 by CNS neurons in adulthood rather than during postnatal development. TSP4 is highly concentrated in synaptic layers in the adult retina and brain, as well as at the mature NMJ.

TSP has recently been identified as a component of the CNS ECM that plays a crucial role in synapse formation *in vitro* and *in vivo*. Using purified cultures of neurons, it was found that neurons in the absence of glial cells can survive and extend neurites, but they form only a few synapses. The number of synapses, however, could be increased by many folds if a culture medium conditioned by factors secreted by glial cells is added to the neurons (Slezak and Pfrieder 2003). A recent report provides evidence that TSP is the astrocyte-derived signal that increases synapses on purified neurons (Christopherson et al. 2005). This study shows that astrocytes *in vivo* and *in vitro* express high levels of TSP1 and 2 mRNA. Addition of purified recombinant TSP2 to cultures of neurons resulted in a dramatic increase in synapse number, similar to the effects of astrocyte-conditioned media (Christopherson et al. 2005), indicating that TSP2 is indeed sufficient to increase synapse number. To determine if TSP2 is a necessary signal from astrocytes for their synapse-promoting ability, TSP2 was removed from astrocyte conditioned media with a TSP2 specific antibody. TSP2 depletion abolished the synapse-inducing activity of astrocyte-conditioned media. These findings indicate that TSP2 is sufficient and necessary for astrocyte-conditioned media to induce synaptogenesis between retinal ganglion cells *in vitro*.

Since TSP1 had a similar synapse-promoting effect as TSP2 and appeared to be expressed *in vivo*, both TSPs may play a role in synaptogenesis *in vivo*. This was verified by analysis of the double KO mice deficient in TSP1 and TSP2. Double KO brains had about 30% less synapses at early postnatal ages than wild-type controls, and this deficit in synapses was maintained into adulthood (Christopherson et al. 2005). Since no significant effect on synapse number was seen in the TSP1 or TSP2 single KOs, it is likely that they may serve redundant functions in the nervous system, each being able to regulate synaptogenesis. How these TSPs send signals to neurons remains to be determined. Since multiple integrins and integrin-associated proteins are among the TSP receptors that are highly localized to synapses in the CNS, signaling of TSPs via integrins is an attractive hypothesis.

6

Synaptic Functions of Reelin

Integrins have also been recognized as receptors for an ECM glycoprotein called reelin, named after the spontaneously occurring *reeler* mouse mutant, which shows abnormal migration of neurons during embryonic development. These deficits result in an outside-in instead of inside-out positioning of migrating neuronal cell bodies in the cerebral cortex. Strikingly, mice deficient

in receptors for reelin, the very low density lipoprotein (VLDL) receptor and apoE receptor 2 (apoER2), and cytoplasmic adaptor protein mDab1, which mediates signaling through VLDL and apoER2 receptors, also display the *reeler* phenotype.

Several studies suggest that reelin together with VLDL and apoER2 receptors are involved in synaptic plasticity (Fig. 1). *Reeler* mice show abnormal LTP in some layers of the hippocampus (Ishida et al. 1994) and even heterozygous *reeler* mice exhibit a decrease of dendritic spine density on cortical and CA1 pyramidal neurons of the hippocampus (Liu et al. 2001). Application of reelin to wild-type hippocampal slices produces a significant increase in LTP (Weeber et al. 2002). Application of receptor-associated protein RAP, a specific and broad-spectrum inhibitor of ligand binding to all low density lipoprotein receptor family members, almost completely blocks LTP (Liu et al. 2001; Weeber et al. 2002). A modest decrease in short-term potentiation is observed in VLDL receptor-deficient mice, although LTP in these mutants is nearly identical to that of the wild-type mice. However, apoER2 mice were strongly impaired in LTP. Importantly, when reelin was applied to slices from VLDL receptor-deficient or apoER2-deficient mice, the LTP-enhancing effect of reelin was abolished. When VLDL receptor- and apoER2-deficient mice were tested in the contextual fear-conditioning learning paradigm, which depends on the hippocampus, both exhibited significant deficits. So, each type of LDL receptor seems to play a role in hippocampal synaptic plasticity and associative memory formation, by signaling downstream of reelin.

A recent study uncovered the underlying mechanisms (Beffert et al. 2005). It provides evidence that apoER2 is present in the postsynaptic densities of excitatory synapses, where it forms a functional complex with NMDA receptors. Reelin signaling through apoER2 markedly enhances LTP through a mechanism that requires the presence of a stretch of amino acid residues in the intracellular domain of apoER2 encoded by a single exon. This exon is alternatively spliced in an activity-dependent manner and is required for reelin-induced tyrosine phosphorylation of NMDA receptor subunits. Mice constitutively lacking the exon perform poorly in learning and memory tasks.

It appeared that this mechanism is also important for maturation of synapses in hippocampal cultures (Sinagra et al. 2005). In this system, chronic blockade of the function of reelin with antisense oligonucleotides or the function-blocking antibody prevents the decrease of NR1/NR2B-mediated whole-cell currents, which is a hallmark of synaptic maturation. Conversely, exogenously added recombinant reelin accelerated the maturational changes in NMDA-evoked currents. Importantly, the change in NMDAR subunits was also blocked by chronic treatment with an inhibitor of the Src kinase signaling pathway or by an antagonist of the low density lipoprotein receptors (Sinagra et al. 2005). Thus, tyrosine phosphorylation appeared to be critical in mediating effects of reelin on synaptic functions. The importance of these

findings is underscored by reduction of reelin expression in psychotic patients and similarity of cellular and synaptic abnormalities observed in these patients and in reelin-deficient mice.

7

Clustering of Glutamate Receptors by Neuronal Pentraxins

In addition to clustering of neurotransmitter receptors via the intracellular postsynaptic scaffold, a mechanism involving aggregation of AMPA receptors through direct interaction with ECM molecules—the complex of neuronal pentraxin NP1 and neuronal activity-regulated pentraxin (Narp or NP2)—has been described (Xu et al. 2003). These two proteins are covalently linked by disulfide bonds to form highly organized complexes, and their relative ratio in the complex is dynamically dependent upon the neuron's activity history and developmental stage. Narp is a secreted protein that is selectively enriched at excitatory synapses on the dendritic shafts of cultured spiny and aspiny hippocampal interneurons, but is not present at excitatory synapses on dendritic spines *in vitro* and *in vivo*. In Narp-transfected cells, the protein interacts with itself, forming large surface clusters that co-aggregate with AMPA receptor subunits (Fig. 1) (O'Brien et al. 1999). Narp molecules co-cluster and co-immunoprecipitate with AMPA receptor subunits GluR1–3, but not with the AMPA receptor subunit GluR4, NMDA receptor subunits NR1 and NR1/2A or kainate receptor subunit GluR6. Expression of dominant-negative Narp mutant protein that prevents its accumulation at synapses led to a marked decrease in the ability of transfected cells to induce GluR1 clusters (O'Brien et al. 2002). Application of exogenous Narp to cultured hippocampal neurons induced clusters of AMPA receptors. Surprisingly, this also led to clustering of NMDA receptors on interneurons, but not on pyramidal cells (Mi et al. 2002). Because Narp does not directly aggregate NMDA receptors, the underlying mechanism seems to involve a recruitment of NMDA receptors via its binding to SAP90/PSD-95, that in turn may associate with the AMPA receptor binding protein stargazin (Mi et al. 2004). As expression of Narp is up-regulated after induction of LTP (O'Brien et al. 1999), it is very likely that deposition of Narp in the ECM will be crucial for expression of LTP in excitatory synapses on interneurons.

Narp is substantially more effective than NP1 in assays of cell surface cluster formation, co-clustering of AMPA receptors, and excitatory synaptogenesis, yet their combined expression results in synergistic effects. These data support a model, in which Narp can regulate the latent synaptogenic activity of NP1 by forming mixed pentraxin assemblies. This mechanism appears to contribute to both activity-independent and activity-dependent excitatory synaptogenesis *in vitro* (Xu et al. 2003), although its relevance to synaptogenesis *in vivo* remains to be demonstrated.

Another potentially interesting link between AMPA receptors and ECM proteoglycans was uncovered in experiments showing that heparin increases the open probability of purified AMPA receptors reconstituted in lipid bilayers (Hall et al. 1996; Sinnarajah et al. 1999). These data suggest that members of the HSPG family may directly interact with AMPA receptors to affect their activity and, possibly, stimulate their aggregation.

8

HB-GAM and N-syndecan in Synaptic Plasticity

Another molecule that binds to heparin is the heparin-binding growth-associated molecule (HB-GAM). It has been implicated in the regulation of neurite outgrowth, axon guidance and synaptogenesis *in vitro* (for a review see Rauvala and Peng 1997). HB-GAM is expressed in an activity-dependent manner (Lauri et al. 1996). In the adult hippocampus, application of HB-GAM inhibited NMDA receptor-dependent LTP, but did not affect L-type VDCC-dependent LTP, that was induced by application of the K⁺ channel blocker tetraethylammonium. This indicates that the action of HB-GAM is limited to NMDA receptor-dependent LTP (Lauri et al. 1998). In HB-GAM-deficient mice, a lower threshold for induction of LTP was observed, which was restored to the wild-type level by application of HB-GAM (Amet et al. 2001). The observation that LTP is attenuated in transgenic mice that over-express HB-GAM is consistent with these findings. These changes in LTP are accompanied by behavioral alterations. Paradoxically, mice over-expressing HB-GAM (with impaired LTP) learn more quickly in the water maze and are less anxious in the elevated plus-maze than wild-type mice, whereas HB-GAM-deficient mice (with a low threshold for induction of LTP) learn less well in the water maze and are more anxious in the elevated plus-maze (Pavlov et al. 2002).

As HB-GAM binds to the HSPG syndecan-3 (N-syndecan), its role in LTP is of great interest. Injection of heparin or removal of heparan sulfate by heparitinase treatment inhibited LTP, showing that LTP depends on endogenous heparan sulfates. The importance of HSPGs is further underscored by recent demonstration that HSPGs in association with polysialylated neural cell adhesion molecule PSA-NCAM regulate synaptogenesis and LTP-induced formation of perforated synapses (Fig. 1) (Dityatev et al. 2004). Syndecan-3 was identified as one of the major HSPGs that is expressed in the hippocampus, and injection of purified syndecan-3 was found to inhibit LTP (Lauri et al. 1999). Mice lacking syndecan-3, on the other hand, exhibited enhanced levels of CA1 LTP and were not responsive to HB-GAM. Behavioral analysis of syndecan-3-deficient mice revealed their impaired performance in hippocampus-dependent learning tasks. These data indicate that syndecan-3 can act as a receptor for HB-GAM and thereby influences synaptic plastic-

ity and hippocampus-dependent memory (Kaksonen et al. 2002). Interestingly, interaction of syndecan-3 with the intracellular cytoskeleton-regulating molecules cortactin and fyn kinase is important for neurite outgrowth, and this interaction is increased after induction of LTP (Lauri et al. 1999). Since recent findings indicate that impaired LTP in HB-GAM over-expressing mice can be rescued by an inhibitor of GABA_A receptors and there is an increase in inhibitory currents in these mutants (Taira, personal communication), it is plausible that HB-GAM signals via syndecan-3 and fyn kinase to promote synaptic strength of excitatory synapses on interneurons and by this mechanism enhances GABAergic inhibition and “restrains” synaptic plasticity.

9

Tenascin-R, GABAergic Transmission and Metaplasticity

Another example of how ECM molecules affect GABAergic transmission is provided by the glycoprotein tenascin-R (TN-R). TN-R is enriched at the nodes of Ranvier and in the perineuronal nets and is essential for assembly of these nets since distribution of net-associated ECM molecules is altered in TN-R-deficient mice (Weber et al. 1999; Bruckner et al. 2000). Furthermore, a recent study revealed rather general changes in the organization of extracellular space in TN-R KO brains, as detected via diffusion measurements (Sykova et al. 2005). Also, TN-R-deficient mutant mice showed impaired CA1 LTP (Bukalo et al. 2001; Saghatelian et al. 2001) and several studies revealed that this abnormality is related to a deficiency in the unusual carbohydrate epitope HNK-1 (a structure containing a 3'-sulfated glucuronic acid, which was first discovered on human natural killer cells—hence the name) carried by tenascin-R. First, mice deficient in glucuronyl-transferase and HNK-1 sulfotransferase, which are the final pathway enzymes in the synthesis of the HNK-1 carbohydrate, showed a similar reduction in CA1 LTP as was found in TN-R-deficient mice (Senn et al. 2002; Yamamoto et al. 2002). Second, reduced LTP in TN-R- and HNK-1 sulfotransferase-deficient mice was accompanied by increased basal excitatory synaptic transmission in synapses formed on CA1 pyramidal neurons (Saghatelian et al. 2001; Senn et al. 2002). Consistent with the *in vitro* data, intrahippocampal recordings *in vivo* revealed an increase in the amplitude of auditory evoked potentials and gamma-oscillations in TN-R KO mice (Gurevicius et al. 2004). Third, the amplitudes of unitary perisomatic inhibitory postsynaptic currents were smaller in TN-R mutants and HNK-1 antibody-treated wild-type mice (Saghatelian et al. 2000, 2001). These data correlated with the results of a quantitative electron microscopic analysis of TN-R-deficient mice, which detected a strong reduction in the density and an abnormal architecture of symmetric perisomatic synapses in the CA1 area of the hippocampus (Nikonenko et al. 2003a). Changes in the density

and spatial arrangement of synaptic vesicles in the synaptic terminals also provided ultrastructural evidence for reduced inhibitory synaptic activity in TN-R mutants.

Mechanisms underlying the action of HNK-1 on GABAergic transmission have been elucidated in experiments with the HNK-1 antibody, which produced a strong and specific reduction in perisomatic inhibitory currents when applied to hippocampal slices (Saghatelian et al. 2000). Antagonists to GABA_B receptors and G-protein-coupled inwardly rectifying K⁺ channels (GIRKs) abolished the effects of the HNK-1 antibody on perisomatic inhibition, indicating that activation of GIRKs via GABA_B receptors is involved in HNK-1 carbohydrate function. Interestingly, synthetic HNK-1 carbohydrate or its peptidomimetic inhibited GABA_B receptor-activated GIRK currents, although not GIRKs directly. As the HNK-1 carbohydrate and its peptidomimetic bind to GABA_B receptors, the results imply that the HNK-1 antibody relieves a constitutive block of GABA_B receptors exerted by endogenous HNK-1 carbohydrate in perisomatic synapses (Saghatelian et al. 2003).

Is the action of HNK-1 pre- or postsynaptic? Infusion of K⁺ channel-blocking Cs⁺ ions into postsynaptic CA1 pyramidal neurons diminished the effects of the HNK-1 antibody on perisomatic inhibitory currents, indicating that the HNK-1 carbohydrate regulates the activation of postsynaptic K⁺ channels (Saghatelian et al. 2003). However, the consequences of this activation impinge on presynaptic GABA release. As elevation of extracellular K⁺ concentration mimicked and occluded the effects of the HNK-1 antibody on inhibitory currents, it is plausible that outflow of K⁺ from postsynaptic cells induces changes in excitability and/or presynaptic machinery. However, influences of other retrograde messengers are not fully ruled out. The functional link between the HNK-1 carbohydrate and TN-R as its cognate "carrier" was directly demonstrated by experiments, in which HNK-1 antibody was applied to hippocampal slices from mice deficient in recognition molecules known to carry the HNK-1 carbohydrate: The antagonistic effect of HNK-1 antibody on inhibitory currents was abolished in TN-R mutant mice, but not in NCAM-deficient mice, indicating that TN-R is the predominant carrier of the HNK-1 carbohydrate that modulates perisomatic inhibition (Saghatelian et al. 2000).

Thus, TN-R/HNK-1 deficiency leads to reduction in GABAergic inhibition and impairment in LTP. A recent study (Bukalo et al. 2005) suggests that the latter is due to changes in the threshold for induction of LTP at these synapses, which was evaluated using pairing of low-frequency presynaptic stimulation with depolarization of postsynaptic CA1 pyramidal cells to -20, -10 and 0 mV. In TN-R KO mice, the threshold was increased by 10 mV in comparison to wild types and this difference appeared between the second and third week of postnatal development, i.e. during maturation of the GABAergic system. To evaluate whether changes in LTP are related to reduced GABA_A and/or increased GABA_B receptor-mediated currents in TN-R KO mice, it was examined whether manipulations of these currents could

lead to a recovery of LTP. Pre-treatment of hippocampal slices with either a GABA_B receptor antagonist CGP54626 or one of the GABA_A receptor agonists, muscimol or zolpidem, restored theta-burst-induced LTP in TN-R KO mice. Zolpidem could also normalize the threshold for pairing-induced LTP. Furthermore, in vivo injection of TN-R KO mice with muscimol completely restored both LTP and levels of excitatory synaptic transmission. These observations provide first evidence that abnormal levels of inhibition in TN-R/- mice cause metaplastic changes in LTP (Bukalo et al. 2005).

10

Tenascin-C and Synaptic Plasticity

It was first recognized that TN-C may be relevant to synaptic plasticity when TN-C was found to be up-regulated in the hippocampus both at the mRNA and protein levels within several hours after induction of LTP in the dentate gyrus (Nakic et al. 1998). In TN-C KO mice, theta-burst stimulation of Schaffer collaterals induced reduced LTP, whereas CA1 LTD was completely abolished (Evers et al. 2002). Recordings of LTP in the presence of nifedipine, an antagonist of L-type VDCCs, did not affect LTP in TN-C-deficient mice, but reduced LTP in wild-type mice to the levels seen in the mutant, implying a link between VDCCs and TN-C in the regulation of synaptic plasticity. Furthermore, chemical induction of a VDCC-dependent LTP in the CA1 region by application of the K⁺ channel blocker tetraethylammonium resulted in impaired LTP in TN-C-deficient mice. As NMDA receptor-mediated responses and three forms of L-type VDCC-independent LTP seemed to be normal in TN-C-deficient mice, all data point to a specific role for TN-C in L-type VDCC-mediated signaling (Fig. 1).

In a parallel study (Strekalova et al. 2002), it was found that TN-C-deficient mice, as well as wild-type mice that had received an intrahippocampal injection of the fragment comprising TN-C FN-like repeats 6 – 8, were impaired in memory recall in a step-down avoidance task. These findings correlated with electrophysiological experiments, which showed a strong reduction of LTP in the CA1 hippocampal area after injection of the fragment. In contrast, the TN-C fragment comprising FN-like repeats 3 – 5 had no effect on LTP and memory. The combined observations not only support the view that TN-C is acutely involved in synaptic plasticity, but also that a particular domain exerts this function. It is noteworthy in this context that the fragment containing FN-like repeats 6 – 8 binds to another ECM molecule, i.e. FN. The fragment, thus, could act via competition with endogenous TN-C, disrupting the targeting of TN-C to the ECM scaffold via FN.

The hypothesis that TN-C affects signaling via L-type Ca²⁺ channels was recently supported by optical measurements of the intracellular Ca²⁺ concentration. A difference between Ca²⁺ signals triggered by theta-burst stimula-

tion in normal physiological solution and in the presence of nifedipine was used as a measure of Ca^{2+} influx via L-type Ca^{2+} channels. The measurements revealed a clear reduction in Ca^{2+} influx in TN-C KO mice (Volgushev and Balaban, personal communication). More work is necessary to dissect the mechanisms of L-type Ca^{2+} channel regulation by TN-C and to elucidate whether this mechanism is related to impaired vibrissotomy-induced plasticity in the somatosensory cortex and numerous morphological cortical abnormalities in TN-C KO mice (Cybulska-Klosowicz et al. 2004; Irintchev et al. 2005).

11

NG2 and Glia-neuron Synapses

NG2 is a single pass transmembrane chondroitin sulfate proteoglycan (CSPG), which has been reported to interact with type VI collagen and therefore acts as a surface ECM receptor (Nishiyama et al. 1991; Nishiyama and Stallcup 1993). It consists of a large extracellular domain comprising two laminin G domains at the N-terminus and has a short cytoplasmic tail of 76 amino acids. The protein is expressed by a class of immature glial cells with a stellate morphology, often referred to as oligodendrocyte progenitor cells (OPC) and by a novel type of postmitotic glial cells (Bergles et al. 2000; Diers-Fenger et al. 2001; Schneider et al. 2001; Butt et al. 2002; Greenwood and Butt 2003). Surprisingly, activation of CA3 pyramidal neurons and Schaffer collaterals resulted in rapid inward currents in NG2-positive OPC from the CA1 region of rat hippocampal slice cultures. Paired stimuli produced larger currents, similar to paired-pulse facilitation of EPSCs in CA1 pyramidal neurons (Bergles et al. 2000). These currents were blocked by AMPA receptor antagonists and indicating that they were mediated by AMPA receptors. The quantal nature of these responses and their rapid kinetics indicated that they were produced by the exocytosis of vesicles filled with glutamate directly juxtaposed to these receptors. Electron microscopic studies revealed synaptic junctions on NG2-positive cells in both young and adult hippocampus (Bergles et al. 2000; Lin and Bergles 2004a). It has recently been found that these synapses are not only glutamatergic but can also be GABAergic (Lin and Bergles 2004b). Furthermore, it became obvious that NG2-expressing cells in the cerebellum receive synaptic input from climbing fibres (Lin and Bergles 2004b; Lin et al. 2005).

An active role of NG2 in the formation of the neuron-glial synapses is suggested by the result of a yeast two-hybrid screen using the cytoplasmic part of NG2 as bait. A PDZ binding motif at the C-terminus of NG2 has been reported to interact with the glutamate receptor interacting protein 1 (GRIP1) (Stegmuller et al. 2003). The authors were able to co-immunoprecipitate NG2, GRIP1 and GluR2 from cultured primary oligodendrocytes, thus suggesting

the existence of a tripartite complex *in vivo*. This finding strongly suggests a role of NG2 in glia-neuronal signaling processes.

12

Lecticans Differentially Contribute to Synapse Formation and Function

A final group of extracellular molecules to be discussed in the context of synaptic functioning are the CSPGs of the lectican family (for a review see Yamaguchi 2000). This family comprises four abundant components of brain ECM: aggrecan and versican as broadly expressed CSPGs and neurocan and brevican as nervous system-specific family members. Because of manifold post-translational modifications each lectican occurs in several isoforms: alternative splicing, differential addition of CS side chains, variable glycosylation and controlled proteolytic cleavage yield a whole plethora of discrete molecular species with slightly different spatial and temporal expression patterns, interaction partners and network-forming properties.

Lecticans can be considered as connectors between neuronal surfaces, where they are able to bind with their C-terminal globular domains, and hyaluronic acid (HA), the central organizing polysaccharide of the ECM, which is bound by the N-terminal globular domains of the CSPGs. Thus, the lecticans together with link proteins, HA and glycoproteins like the tenascins form huge carbohydrate-protein aggregates, e.g. the perineuronal nets and tie them to the cell surfaces (Fig. 2).

First evidence for a plasticity-relevant role of these proteoglycans originates from the pioneering work by Sue Hockfield and colleagues (Zaremba et al. 1989; Lander et al. 1997) who described the activity-dependent regulation of the biosynthesis of the CSPG epitopes CAT-301, -315 and -316, which later turned out to be differentially glycosylated aggrecan isoforms (Matthews et al. 2002).

Versican, also termed PG-M, has a splice variant V1, which is a component of CNS matrix that is expressed rather early during development. It was recently reported to promote the maturation of presynaptic varicosities within retinal arbors innervating the embryonic chick optic tectum (Yamagata and Sanes 2005). Utilizing RNAi techniques the authors demonstrated a selective effect of versican on the shaping of large varicosities.

Neurocan is another typical proteoglycan in the early developing rodent brain. Constitutive neurocan deficiency in mutant mice results in reduced plasticity of Schaffer collateral synapses onto CA1 hippocampal pyramidal neurons *in vitro*, as measured in LTP and GABA blockade paradigms (Zhou et al. 2001). In these mice, no significant differences in basic synaptic transmission and in induction of synaptic potentiation could be detected. However, maintenance of LTP (> 2 hrs after induction) was clearly reduced as compared to wild-type controls. Picrotoxin-induced disinhibition appeared

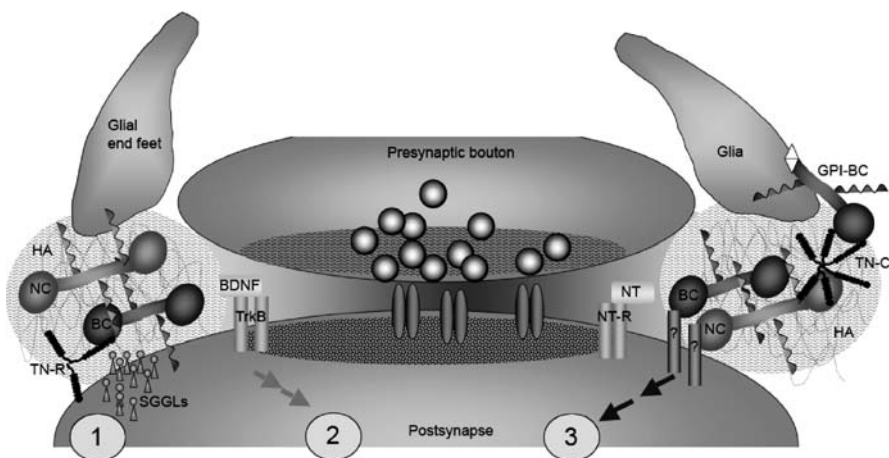


Fig. 2 Hypothetical model that illustrates potential mechanisms for the modulation of synaptic function and plasticity by secreted proteoglycans of the lectican family. Brevican (BC) and neurocan (NC) are secreted into the extracellular space, where they bind to hyaluronic acid (HA), tenascin-C (TN-C), tenascin-R (TN-R) (Aspberg et al. 1997; Hagihara et al. 1999) or sulfated cell-surface glycolipids (SGGLs) (Miura et al. 1999, 2001) to form huge sugar-protein-aggregates, which are concentrated in close proximity to synapses but omit the synaptic cleft. (1) Chemical and/or electrical insulation of synaptic contact sites, maintenance of ion homeostasis, pH and diffusion properties. (2) Low-affinity binding of neurotrophins (NT) like brain-derived neurotrophic factor (BDNF). (3) Putative (peri-) synaptic proteoglycan receptors transducing signals from the ECM to postsynaptic neurons

also to be restricted to the first two hours after application and declined afterwards. Since late LTP phases are characterized by synaptic remodeling, it is plausible to assume a role for neurocan in such processes.

Brevican, also termed BEHAB (brain enriched hyaluronan binding protein), is the closest relative of neurocan and one of the most abundant CSPGs in mature rodent CNS. It is widely distributed in the brain, but highly concentrated in perineuronal nets and in synaptic layers, where it localizes perisynaptically (Seidenbecher et al. 1997). Biochemical fractionation reveals an enrichment of membrane-bound brevican in synaptic fractions (Seidenbecher et al. 2002). Application of anti-brevican antibodies to naive rat hippocampal slices leads to a drastic reduction of CA1 LTP already 30 min after tetanization of the Schaffer collaterals (Brakebusch et al. 2002). This finding is corroborated by LTP studies in brevican-deficient mice. Although basic properties of excitatory and inhibitory synaptic transmission in the mutants are normal, LTP maintenance is dramatically impaired within the same time frame as in the antibody experiment (Brakebusch et al. 2002). The observed LTP defects, however, seem not to be accompanied by learning and memory deficits in the KO mice.

Even though the brevicin antibody should primarily neutralize the core protein it is not completely clear to what extent the plasticity-reducing effect is mediated via the attached CS side chains. Treatment of hippocampal slices with the bacterial enzyme chondroitinase ABC, which removes CS from brevicin and the other CSPGs, led to a 50% reduction in LTP expression. Furthermore, CS digestion also diminished LTD maintenance, although basal transmission as well as short-term plasticity remained unaffected (Bukalo et al. 2001).

Putative mechanisms how lecticans might influence synaptic transmission are discussed in Fig. 2:

- (1) Perisynaptic nets of ECM may insulate synaptic contact sites to prevent spill-over of released neurotransmitters to neighboring synapses or extrasynaptic neurotransmitter receptors. They maintain ion homeostasis, pH and diffusion properties in perisynaptic microenvironments.
- (2) Some proteoglycans are known as low-affinity co-receptors for neurotrophins (NT) like brain-derived neurotrophic factor (BDNF). These soluble factors bind with high affinity to their neurotrophin receptors (NT-R), e.g., TrkB. NT-mediated signal transduction largely influences synaptic transmission. Proteoglycans may contribute to these processes via trapping and concentrating neurotrophins at synapses and presenting them to their cognate receptors.
- (3) An intriguing hypothesis is based on the existence of hitherto not yet characterized proteoglycan receptors, which might actively transduce signals from the ECM to postsynaptic neurons, thus directly influencing synaptic transmission properties. A candidate molecule could be NCAM, a cell adhesion molecule implicated in synaptic plasticity (reviewed in Ronn et al. 1997; Welzl and Stork 2003). Neurocan as well as phosphacan are known to bind to NCAM (for a review see Margolis et al. 1996).

13

Receptor Protein Tyrosin Phosphatase β (RPTP β) and Phosphacan

Phosphacan/RPTP β is a CSPG, which is expressed predominantly in the brain. It occurs in four alternative splicing variants resulting in two transmembrane and two secreted isoforms. The full-length molecule is a receptor protein tyrosine phosphatase (RPTP β long) and consists of a N-terminal carbonic anhydrase-like domain, a FNIII domain, a CS glycosaminoglycan attachment region, a transmembrane region and two intracellular phosphatase domains. A short receptor form (RPTP β short) lacks a large part of the CS glycosaminoglycan attachment region. The two secreted isoforms are termed phosphacan and PSI (phosphacan short isoform) and are the soluble forms of RPTP β long and RPTP β short, respectively (Canoll et al. 1996; Heck et al. 2005). The extracellular domains are known to interact with a number of pro-

teins including members of the Ig-superfamily of cell adhesion molecules, growth factors and extracellular matrix proteins including the tenascins. Phosphacan expression is regulated in an activity-dependent manner, for instance, in the hypothalamic supraoptic nucleus (Miyata et al. 2004). Proteins of the RPTP β family are expressed in close vicinity to synapses, where they are well in place to bridge and stabilize perisynaptic glial processes near synapses via an interaction with the contactin-Caspr complex. This notion is supported by the finding that RPTP β variants are distributed abnormally in contactin-deficient mice (Murai et al. 2002). These mice showed impaired LTD, accompanied by a small increase in synapse density in the CA1 region. Both findings could be due to a reduced number of glial processes that intermingle between synapses.

The involvement of the RPTP β family in synaptic plasticity was further supported by the analysis of RPTP β knockout mice (also termed Ptp α). These mice exhibited enhanced hippocampal LTP in an age-dependent manner, which could be overruled by pharmacological inhibition of the Rho-associated kinase (ROCK), the major downstream effector of Rho (Niisato et al. 2005). Interestingly, this abnormality was accompanied by impairments in the Morris water maze task, suggesting that enhanced LTP is not necessarily beneficial for learning. Thus, it has become clear that the RPTP β family is important for synaptic function through a number of direct and indirect pathways.

14

Matrix Metalloproteases and the Modulation of Perisynaptic Matrix

Matrix metalloproteases (MMPs) are well known for their ability to digest components of the extracellular matrix, such as brevican, aggrecan and agrin (Kuno et al. 2000; Nakamura et al. 2000; VanSaun and Werle 2000). At the NMJ, MMP-3 is expressed and is able to cleave agrin *in vitro* and *in vivo*. Endplates from MMP-3 deficient mice have an increased volume of AChR-stained regions, leaving only small regions devoid of the receptor, and increased agrin immunofluorescence. An increased number and size of junctional folds, and additional ectopic folds accompany changes in expression of agrin and AChR (VanSaun et al. 2003; Werle and VanSaun 2003). MMP-3 can be produced by Schwann cells in its pro-form and is activated via cleavage by the serine protease plasmin (Goldberg et al. 1990). Thus, a signaling cascade might exist, which involves plasmin, plasminogen activator and MMP-3, which regulates removal of agrin from the NMJ and thus shapes NMJ synaptic properties.

In the hippocampus, several MMPs are expressed. Remarkably, expression of MMP-3 and -9 is increased transiently during water maze acquisition in a NMDA receptor-dependent manner. Inhibition of MMP activity with MMP-3 and -9 antisense oligonucleotides and/or MMP inhibitor FN-439

Table 1 Synaptic functions of ECM molecules

Synaptic functions	Agrin	Laminins	Integrin ligands	Thrombo-Spondins	Reelin	Neuronal pentraxins	HSPGs	HB-GAM	Tenascin-R	Tenascin-C	Versican
Synapse formation											
Presynaptic assembly		+		+							+
Postsynaptic assembly	+					+					
Alignment of pre- and postsynapses		+									
Synapse maturation			+		+						
Induction of synaptic plasticity											
Activity of NMDARs			+								
Activity of L-type Ca ²⁺ -channels			+								+
GABAergic inhibition								+		+	
Expression of synaptic plasticity											
Activity of AMPARs			+				+				
Recycling of AMPARs			+								
Recycling of presynaptic proteins		+									

Plus (+) indicates involvement of a molecule in a process.
Abbreviations: HSPG, heparin sulfate proteoglycan; HB-GAM, heparin-binding growth-associated molecule; AMPAR, AMPA-type glutamate receptor; NMDAR, NMDA-type glutamate receptor.

impaired LTP and acquisition of spatial learning. The learning-dependent changes in MMP expression were also shown to modify the stability of the actin-binding protein cortactin, which plays an essential role in regulating the dendritic cytoskeleton and synaptic efficiency (Meighan et al. 2006). The importance of regulated ECM remodeling by MMP in the CNS is further supported by a study of mice deficient in the tissue inhibitor of metalloproteinases-1 (TIMP-1), which display an impairment in olfactory maze learning (Jourquin et al. 2005).

Conclusions

Taken together, a fascinating picture of complex contributions of ECM molecules to the genesis, function and plasticity of peripheral and CNS synapses has emerged during the last 15 years (Table 1) (Sanes and Lichtman 2001; Dityatev and Schachner 2003). This appears to be a rather short time, if one considers the sparse knowledge about molecular components, organizing principles, and binding receptors of neural ECM 15 years ago. Now we can conclude that brain matrix components are in fact much more than just a passive microenvironment repellent for neurite outgrowth to stabilize the *status quo* of mature CNS architecture but a highly dynamic network, which intensely communicates with glial and neuronal cells and contributes to neural synaptic transmission. Thus, presently, chemical synapses may be considered as a structural and functional “Quadrige” of presynaptic terminal, postsynaptic element, glial endfeet infiltrating synaptic contact sites and a highly specialized perisynaptic ECM (Fig. 2).

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Electrical Synapses – Gap Junctions in the Brain

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Abstract In the nervous system, interneuronal communication can occur via indirect or direct transmission. The mode of indirect communication involves chemical synapses, in which transmitters are released into the extracellular space to subsequently bind to the postsynaptic cell membrane. Direct communication is mediated by electrical synapses, and will be the focus of this review.

The most prevalent group of electrical synapses are neuronal gap junctions (both terms are used interchangeably in this article), which directly connect the intracellular space of two cells by gap junction channels. The structural components of gap junction channels in the nervous system are connexin proteins, and, as recently identified, pannexin proteins.

Connexin gap junction channels enable the intercellular, bidirectional transport of ions, metabolites, second messengers and other molecules smaller than 1 kD. More than 20 connexin genes have been found in the mouse and human genome. With the cloning of connexin36 (Cx36), a connexin protein with predominantly neuronal expression, the biochemical correlate of electrotonic transmission between neurons was identified. We outline the distribution of Cx36 as well as two other neuronal connexins (Cx57 and Cx45) in the nervous system, describing their spatial and temporal expression patterns. One focus in this review was the retina, as it shows many and diverse electrical synapses whose connexin components have been identified in fish and mammals. In view of the function of neuronal gap junctions, the network of inhibitory interneurons will be reviewed in detail, focussing on the hippocampus.

Although *in vivo* data on pannexin proteins are still restricted to information on mRNA expression, electrophysiological data and the expression pattern in the nervous system have been included.

1

Introduction

Communication between cells, particularly those of the nervous system, is essential for the existence and survival of any living animal. In the central nervous system, the essential feature of neuronal cell interaction is a high operational speed, which is achieved by synapses.

Synapses can mediate neuronal communication by two means: *indirect transmission*, i.e. chemical synapses acting via neurotransmitter release, or *direct transmission*, i.e. electrical synapses acting via ion, metabolite, and second messenger transmission between cells. The vast majority of electrical

synapses are gap junction channels, the structural correlate of which are *connexin* proteins, and also *pannexin* proteins (Sect. 6.2), the latter being more and more recognized in their channel-forming capacity.

Connexin gap junctions are formed by the apposition of two contacting hemichannels, each in the membrane of one cell. The components of one hemichannel, the connexon, are six transmembrane proteins named connexins (Kumar and Gilula 1996). To date, 20 and 21 connexin genes have been identified in the mouse and human genome, respectively (Willecke et al. 2002; Sohl et al. 2004). Most, but not all, of the mouse connexin genes correspond to human orthologous connexin sequences (Sohl et al. 2004). Connexins (Cx) are generally named after their approximate molecular weight, i.e. Cx32 for a 32 kD protein. This family of proteins possesses several connexin-specific characteristics, for instance four transmembrane domains, two extracellular

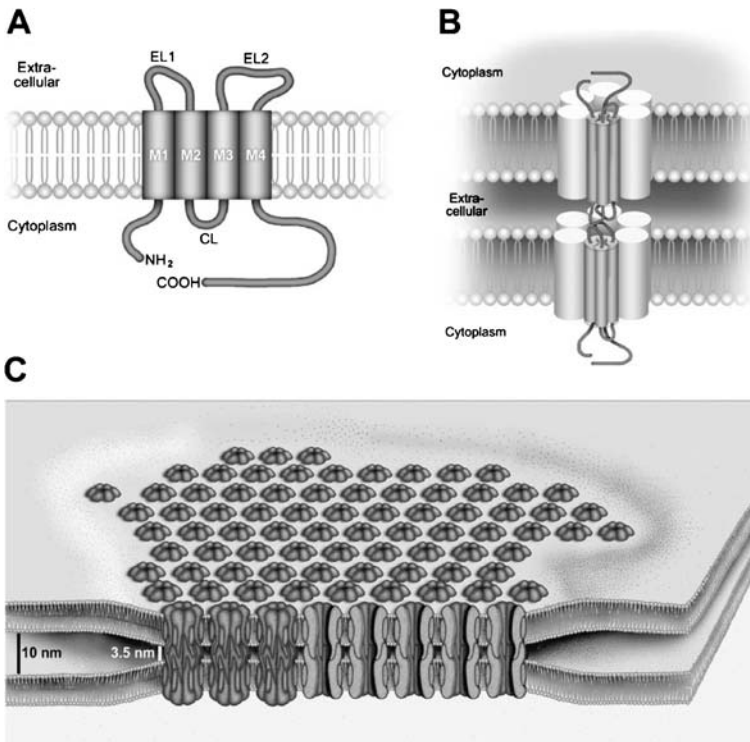


Fig. 1 Schematic representation of gap junctions and their connexin constituents. **a** Connexin protein with cytoplasmic C- (COOH) and N- (NH₂) terminus, four transmembrane domains (M1–M4), two extracellular (EL1 and EL2) and one cytoplasmic loop (CL) domain. **b** Single gap junction channel, formed by close apposition of two hemichannels, each assembled by six connexin proteins. **c** Gap junction plaque, characterized by the reduced distance between cell membranes (3.5 nm within the gap junction area)

loops, and cytoplasmic C- and N-termini (Kumar and Gilula, 1996) (Fig. 1a). The two cytoplasmic domains, particularly the C-terminus, were recently shown to be important for signal transduction, phosphorylation events and association with kinases, tight junction proteins and the cytoskeleton (Giepmans, 2004). The N-terminal domain, at least in the Cx36 protein, has been implicated in the determination of its subcellular localization (Zoidl et al. 2002). Connexons (Fig. 1b) may have only one type of connexins (homomeric connexons), or several (heteromeric), and the gap junction channel can be formed by two identical (homotypic) or different (heterotypic) connexons. The most common assembly of gap junction channels is the gap junction plaque (Fig. 1c), characterized by the reduced space between membranes of contacting cells. However, additional gap junction formations have recently been described in the retina (Rash et al. 2004).

Gap junction channels allow the bidirectional intercellular passage of molecules smaller than 1 kD, thereby mediating ionic and metabolic communication between directly connected cells. With their speed, simplicity and reciprocity, gap junctions can fulfil very specialized tasks in neuronal circuits, which are different from those accomplished by chemical synapses. Gap junctions in the brain show distinct spatial and temporal expression patterns. In neuronal cells, the abundance of gap junctions and their implication for neuronal circuits has only been recognized recently. Electrical synapses are now being extensively examined in mammals, and advances in electrophysiology, transgenic animal technology, cell imaging, and ultrastructural techniques (freeze-fracture replica immunolabelling, FRIL) are allowing the detailed analysis of expression and function of connexins in developing and mature nervous system.

2

Gap Junctions in Development

Connexin gap junctions are prevalent during the early phase of neurogenesis (prenatal phase) (Dermietzel et al. 1989) while chemical synapses emerge in the postnatal phase (Blue and Parnavelas, 1983; Kandler and Katz, 1998). Apparently, both modes of communication serve for different functions, and it is to be expected to find them in different spatial or temporal settings. Direct intercellular transfer of molecules can be detected from the eight cell stage embryo onwards (McLachlin and Kidder, 1986), and gap junction-forming proteins (i.e. connexins) are already present at the zygote stage (Barron et al. 1989). In general, connexin-containing gap junctions allow the intercellular exchange of ions (electrical coupling) and transmission of biologically active molecules (metabolic coupling) between adjacent cells in multicellular organisms. During early cortical development, gap junctions are likely to mediate neuronal communication by *metabolic interaction*. Significant changes hap-

pen during the first two weeks of postnatal development, and as the nervous system matures, gap junctional communication is to some extent replaced by chemical synapses. However, the expression of certain connexin proteins is maintained, and in mature neurons their *electrical language* becomes important in the coordination of neuronal activity, mediating synchrony and network oscillations (Galarreta and Hestrin, 2001; Gibson et al. 2005).

The involvement of connexin gap junctions has been demonstrated at several stages of early cortical development and was shown to be crucial for various developmental landmarks (Dermietzel and Meier, 2005). During early stages of corticogenesis, gap junctional coupling was demonstrated between cells of the ventricular zone, including neural precursors and radial glial cells, which later advance to a columnar organization (Lo Turco and Kriegstein, 1991). Both neural precursors and radial glia cells express Cx26 and Cx43 and these gap junction proteins might therefore participate in metabolic coupling between neocortical precursors. Intriguingly, an inverse correlation between connexin expression and cell proliferation has been described, suggesting that cell cycle and connexin expression might be interdependent.

With development of a complex multilayered cortical structure, prominent amendments happen in the molecular make-up of neural cells: The cellular distribution of connexin proteins becomes characteristic for certain cell types (see below). Immature cells, for instance neural progenitor cells, express connexin proteins differently from their lineage-separated progeny, i.e. neuronal and glial cells.

3

Gap Junctions in Glial Cells

Although the term “electrical synapse” refers primarily to neuronal cell interaction, gap junctions being the structural equivalent of electrical synapses are not only observed on neuronal cells but also on most other cell types in the CNS (Fig. 2). We will therefore provide a short summary of glial connexins in the adult central nervous system. The connexin proteins expressed by macroglia cells include Cx26, Cx30, and Cx43 in astrocytes, and Cx29, Cx32, and Cx47 in oligodendrocytes (Fig. 2).

In oligodendrocytes, Cx32 expression – both spatial and temporal – was shown to coincide with maturation (Belliveau et al. 1991; Belliveau and Naus, 1995), and its expression is maintained in myelin-forming oligodendrocytes (Dermietzel et al. 1989; Micevych and Abelson, 1991; Belliveau and Naus, 1994, 1995; Giaume and Venance, 1995; Scherer et al. 1995; Spray and Dermietzel, 1995). In mature oligodendrocytes, two additional connexin proteins are present: Cx29 and Cx47 (Altevogt et al. 2002; Li et al. 2002; Menichella et al. 2003; Nagy et al. 2003; Odermatt et al. 2003; Kleopa et al. 2004; Nagy et al. 2004). The developmental expression profile of connexins in oligoden-

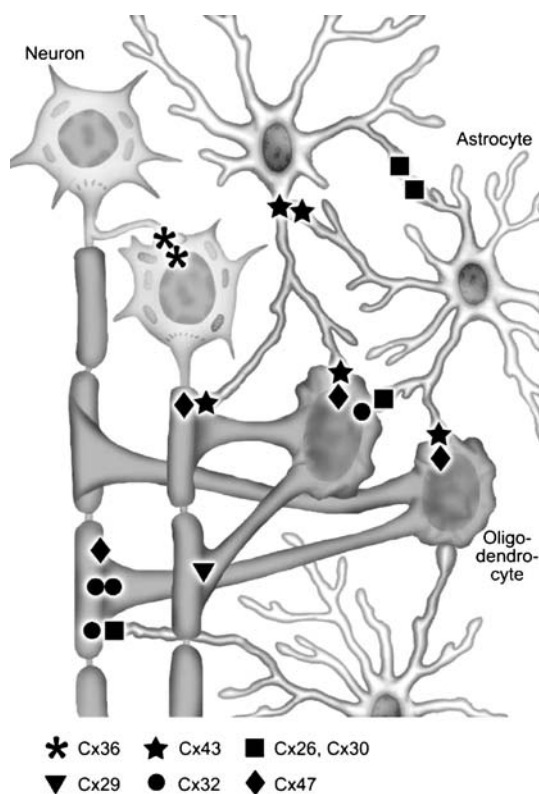


Fig. 2 Schematic representation of connexin expression in astrocytes, oligodendrocytes and neurons. Connexin36 gap junctions in the brain occur between neurons only. Astrocytes form gap junctions consisting of Cx43, Cx26 and Cx30, whereas oligodendrocytes express Cx29, Cx32 and Cx47, with each of these proteins forming gap junctions with distinct partner connexins. The exception is Cx29, the coupling partner of which is still unknown

drocytes parallels that of myelin proteins (Altevogt et al. 2002). Expression of Cx45 has been demonstrated in developing oligodendrocyte precursors, however, studies on Cx45-transgenic animals seem to exclude its expression in mature oligodendrocytes (Maxeiner et al. 2003).

Intriguingly, gap junctions between oligodendrocytes themselves are rarely if ever observed (Massa and Mugnaini, 1982; Waxman and Black, 1984; Rash et al. 2001a). However, oligodendrocytes are integrated into the astrocytic network by gap junctional communication, creating a “panglial syncytium” (Massa and Mugnaini, 1982) consisting of heterotypic channels. It has been suggested that oligodendrocyte Cx32 associates mainly with astrocytic Cx26, whereas oligodendrocyte Cx47 associates with astrocytic Cx43 and Cx30 (Rash et al. 2001a,b; Altevogt and Paul, 2004).

There is also extensive astrocyte–astrocyte coupling observed in the glial syncytium, and mature astrocytes were shown to express a number of con-

nexin proteins, including Cx43, Cx30 and Cx26 (Nagy and Rash, 2000). These three connexin proteins have been identified in astrocytes of adult rat brain and spinal cord and were ultrastructurally identified as astrocytic gap junctions by immunolabelling on freeze-fracture replicas (Nagy and Rash, 2000). Studies on Cx43-deficient astrocytes also imply that the pattern of connexin expression can be modulated: In Cx43-deficient cells, the additional expression of Cx40, Cx45 and Cx46 was detected by RT-PCR (Dermietzel et al. 2000a).

4

Challenges in Expression Analysis of Gap Junction Proteins

Quantitative and qualitative changes of connexin expression were demonstrated under certain developmental and pathological circumstances (see above). Thus, analysis of gap junctional coupling can only provide a depiction of the actual connexin expression, irrespective of a putative diverse molecular make-up at different developmental stages or under the influence of pathological conditions including de- and regeneration. Examples of pathological changes in connexin expression include an increase of Cx46 protein in Schwann cells during peripheral nerve regeneration (Chandross et al. 1996), neuronal expression of Cx45 (increase) and Cx36 (decrease) upon kainate treatment (Condorelli et al. 2003), increased expression of Cx32 and Cx36 in CA1 neurons after global ischemia (Oguro et al. 2001), and the expression of Cx30 in apoptotic neuronal cells after kainate-induced seizures (Condorelli et al. 2002). One example of temporary developmental expression is the transient coupling of developing muscle fibres. In the chick, the earliest developmental stage at which Cx36 mRNA was detected by *in situ* hybridization was embryonic day 2 (i.e. HH 13). During embryonic development, expression of Cx36 mRNA was assigned to myotomes (co-localization with MyoD and FGF-8), whereas a few days later, Cx36 expression was also detected in limb muscles (co-localization with Pax3 and MyoD). In view of skeletal muscle being one of the few tissues that do not show gap junctional communication in the adult animal, the expression of Cx36 during development seemed somewhat surprising. However, several studies have investigated the occurrence of gap junctions ultrastructurally, and from these studies it became evident that developing skeletal muscle cells are indeed initially coupled through gap junctions, but with the formation of muscle fibres become uncoupled (Rash and Staehelin, 1974; Keeter et al. 1975; Kalderon et al. 1977; Schmalbruch, 1982).

In the nervous system, extensive cell branching, long cellular processes, and the intermingling of various cell types impose an additional challenge in gap junction research. Particularly, the assignment of a certain connexin to a specific cell or cell type turned out to be a general problem, as the cellular identity is often obscured and cannot be resolved due to technical restrictions, for instance the limit of resolution. In addition, the detection

of connexin mRNA does not necessarily reflect its translation into protein (Traub et al. 1998; Condorelli et al. 2003); the presence of connexin proteins does not automatically result in junctional conductance as observed in the case of Cx29 (Altevogt et al. 2002).

With more than 20 isoforms of connexin genes, another problem became evident: Any technique based on binding of antibody to antigen (i.e. immunohistochemistry) or on hybridization of probe to mRNA (i.e. in situ hybridization) involves the risk of cross-reactivity between different connexin isoforms. Merely connexin-deficient animals provide unambiguous negative controls for the specificity of antibodies and in situ hybridization probes. The generation of transgenic animals in which the connexin coding sequence has been replaced by reporter genes allows for easier identification and interpretation of the expression profiles of connexins (Sohl et al. 2004). Using the transgenic technique, the reassignment of cellular expression sites has already been achieved in some cases, for instance for Cx45 and Cx47 (Maxeiner et al. 2003; Odermatt et al. 2003). However, every technique has its limitation, and in the case of transgenic animals some expression sites might not be detected because of weak promotor activity or a generally low abundance of the translated product in heterozygous animals. The only way to overcome the described difficulties is the application of a broad spectrum of techniques, including transgenic animals (Meier et al. 2002), and approaches that combine microsampling of tissue with molecular biological methods (Weickert et al. 2005).

5

Some History on Electrical Synapses

In general, synapses can be defined as specialized sites of functional interaction between neurons. As neuronal gap junctions comprise defined areas of neuronal membranes, provide for direct cell-to-cell contact, and enable the exchange of molecules between neurons, they fulfil the requirements for being designated synaptic contacts. Initially, their predominant function was thought to be the intercellular transport of ions, and neuronal gap junctions were therefore named “electrical synapses”. Although this terminology also includes a number of junctional specializations that are not gap junctions (Bennett, 1977; Faber and Korn, 1989; Jefferys, 1995), we will use the terms “neuronal gap junction” and “electrical synapse” synonymously.

The concept that neurons communicate electrically originated from pharmacological, ultrastructural, and physiological observations described more than 50 years ago (Bennett, 1997; Zoidl and Dermietzel, 2002; Connors and Long, 2004). Nevertheless, the tug-of-war for the acknowledgement and acceptance of electrical versus chemical synapses lasted for several decades. Application of computational and molecular methods and technical advances in ultrastructural analysis have provided new insights into the mechanisms

of synaptic transmission, and novel ideas on the impact and importance of neuronal gap junctions have been brought forward.

In the 1950s, the first compelling evidence for the presence of electrical synapses was gathered by studies on lower vertebrates. Initially, electrotonic transmission was identified between segments of crayfish cord giant fibres (Furshpan and Potter, 1957, 1959; Grundfest, 1959). Watanabe (Watanabe, 1958) demonstrated that neurons in the cardiac ganglion of the mantid shrimp, *Squilla*, are electrically coupled – and he suggested that coupling was responsible for synchronous firing of these cells, a hypothesis that would be proven right about 40 years later (see below). In the study by Bennett and co-workers (Bennett et al. 1959), electrical synapses were for the first time identified ultrastructurally in supramedullary neurons of a teleost fish (the pufferfish), and these were also shown to fire synchronously. Because of these findings, the co-existence of electrical and chemical synapses became generally accepted, however, the functional impact of electrical synapses was still attributed to the nervous system of lower vertebrates. In consequence, electrical synapses were thought to represent primitive phylogenetic devices for intercellular neuronal communication that have been abandoned in mammals. This hypothesis had to be revised with the identification of electrotonic transmission in higher vertebrates: In the 1970s, several areas with gap junction-mediated electrical transmission were defined in the mammalian central nervous system, including the olfactory bulb (Pinching and Powell, 1971), neocortex (Sloper, 1972; Peinado et al. 1993), the mesencephalic nucleus (Baker and Llinas, 1971), inferior olive (Llinas et al. 1974) and the cerebellum (Sotelo and Llinas, 1972).

With the cloning of Cx36, the impact of neuronal connexins in the mammalian central nervous system has become much more evident (see below). With innovative methods like freeze-fracture replica immunolabelling, the astonishing abundance of electric synapses begins to be unravelled (Rash et al. 2000, 2001b). However, in some cell types known to be electrically coupled, Cx36 is essentially absent. With the identification of pannexin proteins and the demonstration of their junctional properties, the pannexin family of gap junction proteins moved into the focus of electrical synapse research.

6

Gap Junctions in Neurons – Electrical Synapses

6.1

Connexin Gap Junctions

Of all connexins expressed in cells of the nervous system, only few can be assigned to neurons – and for some others, their cellular correlate is still of debate. At least ten of the mammalian connexins are abundant in the cen-

tral nervous system, however, there seems to be a prevailing separation of neuronal and glial connexins. Hardly any of the “glial” connexins described above are expressed in mature neuronal cells (Rash et al. 2001a,b; Odermatt et al. 2003), although some might be present in neurons during development or under pathological conditions (Nagy et al. 2001; Condorelli et al. 2003; Honma et al. 2004). Intriguingly, in brain areas displaying high electrical coupling, i.e. hippocampus and retina, neurons were devoid of any of the known connexin proteins, which were cloned before 1998. This apparent discrepancy was beginning to be resolved with the discovery of a novel family of connexin proteins, whose expression was attributed predominantly to neurons of the central nervous system.

Cloning of this neuronal subfamily of connexin genes commenced in fish with the identification of Cx35 (O’Brien et al. 1996, 1998; White et al. 1999), which was followed by the cloning of its orthologue, Cx36, in mouse (Condorelli et al. 1998; Sohl et al. 1998) and man (Belluardo et al. 1999). Molecular analysis revealed that Cx35 and Cx36 differ from other connexins by the insertion of a single intron interrupting the coding region, thus placing Cx35/Cx36 in a new subgroup of gap junction proteins, designated the γ -group (Condorelli et al. 1998; O’Brien et al. 1998; Sohl et al. 1998). Initial electrophysiological studies (Srinivas et al. 1999) provided first functional evidence for Cx36 being a main player of electrotonic synapses in neurons.

6.1.1

Spatial Expression of Cx36

The spatial expression of Cx36 was studied by in situ hybridization (Condorelli et al. 1998; Belluardo et al. 1999), Northern blot analysis (Sohl et al. 1998; Al-Ubaidi et al. 2000), RT-PCR (Belluardo et al. 1999; Srinivas et al. 1999), immunolabelling at the light and electron microscopic level (Belluardo et al. 2000; Rash et al. 2000; Teubner et al. 2000; Meier et al. 2002), and in transgenic animals expressing reporter genes under the Cx36 promotor (Deans et al. 2001; Degen et al. 2004). In concurrence, Cx36 is widely expressed in neurons of the central nervous system. High expression levels were found in the following brain regions: olfactory bulb, spinal cord, dorsal root ganglia, brainstem nuclei, cerebellum, thalamus and hypothalamus, septum, basal ganglia, basal forebrain, piriform cortex, amygdala, pineal gland, inferior olive, hippocampus, cortex and retina (Condorelli et al. 2000; Connors and Long, 2004). Further proof of the neuronal expression of Cx36 came from primary cell cultures, in which Cx36 mRNA was identified in neurons but in neither micro- nor macroglial cells (Condorelli et al. 2000).

Despite its predominantly neuronal expression, Cx36 was also detected in tissues outside the nervous system. Using transgenic mice expressing lacZ under the Cx36 promotor, Degen et al. (2004) were able to demonstrate Cx36 expression in the adrenal medulla, and in β -cells of the pancreas, a find-

ing previously also reported on the basis of immunohistochemical results (Serre-Beinier et al. 2000). Pancreatic expression was also confirmed in chick embryos, where Cx36 expression is initially restricted to the mesoderm and its derivatives (Berthoud et al. 2004).

6.1.2

Temporal Expression of Cx36

Concerning the temporal expression of Cx36 in the nervous system, neurons in the embryonic and early postnatal rat cortex were shown to be extensively coupled via gap junctions (Connors et al. 1983; Lo Turco and Kriegstein, 1991; Peinado et al. 1993; Rorig and Sutor, 1996; Rozental et al. 1998), with first detection of Cx36 mRNA in the central nervous system at embryonic day 9.5 in the mouse (Gulisano et al. 2000). High levels of Cx36 are expressed in the perinatal period, reaching a peak of expression at P7 (Sohl et al. 1998). According to Belluardo et al. (2000), the entire neuronal population of certain brain areas is expressing Cx36 during early postnatal development, irrespective of their Cx36 expression in later life. After P7, Cx36 expression shows a progressive decline to adult levels (Sohl et al. 1998; Belluardo et al. 2000). This developmental decrease also parallels the decline of dye coupling between neurons of the rat neocortex (Connors et al. 1983; Rorig et al. 1995; Rorig and Sutor, 1996).

6.1.3

Cx36 and Neuronal Network Oscillations

Nevertheless, Cx36 expression persists in some neuronal subpopulations throughout life – and these expression sites seem to have major influence on neuronal network function. In the adult central nervous system, gap junctional coupling seems to be prevalent between interneurons that influence or entrain networks of principal cells to oscillate. Synchronization of neuronal ensembles occurring at different frequency bands [θ waves 5–10 Hz (Buzsaki et al. 1983; Buzsaki, 2002), γ oscillations 30–80 Hz (Singer and Gray, 1995; Steriade et al. 1996; Traub et al. 1998), and fast spiking oscillations/ripples 100–200 Hz (Ylinen et al. 1995)] has been implicated to underlie a number of cognitive processes including cognition, learning, memory and perception (Gray and Singer, 1989; Miltner et al. 1999; Rodriguez et al. 1999). There are several lines of evidence supporting the notion that electrical synapses are crucially involved in the establishment and perpetuation of these oscillatory networks (Buzsaki et al. 1992; Traub et al. 1996; Buzsaki, 2001; Buzsaki and Draguhn, 2004). With the identification of the neuronal expression of Cx36, the molecular correlate of electrical synapses had been identified, and, as cell-type specific functions seem likely, we will address the expression and function of Cx36 with respect to neuronal subtypes.

Of all adult neurons expressing Cx36, **gamma-aminobutyric acid (GABAergic) interneurons** comprise the population described best, based on morphological and electrophysiological evidence. In several brain regions, dye coupling and synchronization of inhibitory interneurons had previously been demonstrated (Michelson and Wong, 1994; Gulyas et al. 1996). In addition, GABAergic interneurons were shown to be involved in oscillatory neuronal activity, and this effect was clearly mediated by gap junctions (Jefferys et al. 1996; Galarreta and Hestrin, 1999; Gibson et al. 1999). With the identification of Cx36 and its localization in GABAergic interneurons, evidence grew that Cx36 might be the crucial gap junction protein mediating oscillatory behaviour.

In the hippocampus, the majority of Cx36-positive cells are GABAergic interneurons of the CA1 and CA3 region (Belluardo et al. 2000), a finding that was also confirmed by detection of a reporter gene product (Deans et al. 2001; Degen et al. 2004). In the subiculum, i.e. the region between cornu ammonis and the entorhinal cortex, as many as 81% of parvalbumin-positive neurons express Cx36 (Belluardo et al. 2000). The neuronal expression of Cx36 was initially confirmed by co-localization with the neuronal marker protein NeuN, and further classification of neurons demonstrated the presence of Cx36 in parvalbumin-expressing neurons, a distinct population of GABAergic neurons (Belluardo et al. 2000). This observation is in agreement with the ultrastructural detection of gap junctions on parvalbumin-positive GABAergic neurons in the rat hippocampus (Katsumaru et al. 1988) and striatum (Kita et al. 1990). In the hilus of the hippocampus, dendro-dendritic gap junctions have been described in interneurons (rat and guinea-pig). In addition, Cx36 expression was also demonstrated for parvalbumin-negative neurons (Belluardo et al. 2000). This is consistent with the finding that coupling of other neuron-types, for instance somatostatin-immunopositive neurons and low threshold spiking interneurons, is also mediated by gap junctions.

The evidence for Cx36 in principal pyramidal cells of the hippocampus is more ambiguous. Despite detection of Cx36 mRNA in pyramidal cells of the CA3 region in the hippocampus and expression of a reporter gene in pyramidal cells of the CA3 region (Deans et al. 2001), Cx36 protein could not be detected by immunohistochemistry (Belluardo et al. 2000). Initial reports on immunohistochemical labelling of Cx36 in pyramidal cells (Teubner et al. 2000) have later been questioned upon application of those antibodies to tissue derived from Cx36-deficient animals, demonstrating non-specific binding (Meier et al. 2002). Possible explanations for the divergent detection of mRNA and protein include (a) that Cx36 protein expression might be below the limit of detection, and (b) that riboprobes might be less specific than the antibody, thereby possibly cross-reacting with other homologous gap junction proteins yet to be identified. Puzzling data were also provided by studies of neuronal dye coupling, which demonstrated that pyramidal neurons of CA3, but also of

the CA1 region, are extensively coupled, indicating that gap junctional coupling occurs despite the absence of Cx36 protein (see below).

In summary, consensus of the expression analysis has been that Cx36 is strongly expressed in pyramidal cells and interneurons during early postnatal development, but then disappears from the majority of cells in the pyramidal layer (Deans et al. 2001), and is primarily present in interneurons in adults.

Studies on connexin protein expression and formation of gap junction plaques experienced a major advancement by development of the technique of freeze-fracture replica immunogold labelling (FRIL) (Fujimoto, 1995; Rash et al. 1998) as this technique allows the simultaneous detection of immunolabelled integral membrane proteins and the identification of gap junctional ultrastructure and cell type. Employing the FRIL-technique, Cx36-containing gap junction plaques were exclusively identified in neurons of brain and spinal cord (Rash et al. 2000, 2001b; Rash et al. 2001a), and neurons were postulated to share gap junctions only with other neurons, but not with glial cells (Rash et al. 2001b). In several brain areas the ultrastructural identification of Cx36 gap junctions is in agreement with previous mRNA localization, including the nuclear complex of the inferior olive, retina, olfactory bulb and spinal cord (Rash et al. 2000).

The first pioneering work of Traub and co-workers provided a theoretical basis for the impact of few and dispersed gap junctions in neurons. Using computer simulations, this group was able to study the mechanisms of various forms of oscillations in the hippocampus (Traub et al. 1996, 1999, 2001, 2003; Traub and Bibbig, 2000). In these models, electrical synapses were placed at axonal processes of inhibitory neurons, thereby generating sites of axo-axonal contact. These gap junctions were shown to mediate high frequency oscillations of 100–200 Hz, termed fast pre-potentials or ripples (Traub and Bibbig, 2000). The most surprising detail in these models was the demonstration of very few gap junctional channels being required to result in high frequency oscillations. The theoretical findings obtained from these simulations corroborated the observation that gap junction uncoupling agents abolish both ultrafast ripples and γ -frequency oscillations in neuronal networks of the hippocampus (Draguhn et al. 1998; Schmitz et al. 2001).

However, fundamental experimental evidence for Cx36 being the structural correlate of neuronal network oscillations was first provided by electrophysiological studies on Cx36-deficient animals, assessing functional consequences of impaired electrical signalling on neuronal network activity (Deans et al. 2001; Hormuzdi et al. 2001). Interestingly, deletion of the Cx36 gene affected only a subset of oscillatory activities, with others being left unchanged: The most robust effect in the Cx36-deficient mouse was the lower amplitude of γ -frequency oscillations (Deans et al. 2001; Hormuzdi et al. 2001), a component which was previously shown to depend on the presence of electrical synapses (Traub et al. 2000). Disturbance of oscillations in the γ -frequency band resulted in a reduced coherence of inhibitory input onto

pyramidal cells as well as depolarized interneurons of the stratum pyramidale (Hormuzdi et al. 2001). This major impact can be explained by the fact that single hippocampal inhibitory neurons innervate hundreds of principle neurons (Somogyi et al. 1983; Sik et al. 1995; Halasy et al. 1996). The study by Deans et al. (2001) revealed the absence of electrical coupling and spikelets in low threshold spiking types of inhibitory interneurons, which are known to be connected by electrical synapses (Gibson et al. 1999). These findings were also supported by *in vitro* results as cultured parvalbumin-positive dentate gyrus basket cells generally displayed gap junctional coupling. However, upon ablation of the Cx36 gene reciprocal coupling was absent (Venance et al. 2000), and identical observations were made on GABAergic interneurons of the hippocampal CA3 stratum oriens (Hormuzdi et al. 2001).

Fast oscillations occur mostly as sharp wave–ripple complexes in which the sharp wave represents synaptic excitation of CA1 pyramidal cells by a massive, synchronous input from CA3 via the Schaffer pathway (Buzsaki et al. 1992; Ylinen et al. 1995; Draguhn et al. 1998). Ripples were assigned to rapid coupling potentials of pyramidal neurons, which were responsive to a number of agents affecting gap junctions (Draguhn et al. 1998). Schmitz et al. (2001) have taken these studies a step further and showed that the gap junctions mediating this fast spiking rhythm are located between axons of principal cells, thus being axo-axonal (Schmitz et al. 2001). In the study by Hormuzdi et al. (2001), ripples were still present in Cx36-deficient tissue and appeared to be unaffected by the absence of Cx36. However, when the effect Cx36 imposes on ripples was investigated in detail, Maier et al. (2002) demonstrated in a stringent *in vitro* analysis of slice preparations a reduction in frequency and the occurrence of spontaneous sharp waves and ripples in Cx36-deficient mice compared to wild-type animals.

Taken together, expression data and functional analysis contributed to the finding that Cx36 gap junctions are present on inhibitory interneurons and are crucial components of γ -frequency oscillations. However, their implication in slower frequency θ waves and high frequency ripples is still uncertain. As the aforementioned data were collected from slice cultures, *in vivo* analysis might provide less ambiguous evidence. The first *in vivo* studies have meanwhile been performed (Buhl et al. 2003) and present electrophysiological analysis of running or sleeping animals. In this study, γ -frequency oscillations were selectively impaired in Cx36-deficient mice, whereas neither θ waves nor high frequency ripples were affected *in vivo*. Further functional implications of Cx36 have been obtained by investigation of motor and cognitive behaviour: Motor performance was not affected in Cx36-deficient animals (Kistler et al. 2002; Frisch et al. 2005), however, these animals were incapable of distinguishing between newly presented and known old objects in object recognition tasks (Frisch et al. 2005).

In addition to the hippocampus, GABAergic interneurons are also encountered in the cerebral cortex, thalamus, striatum and cerebellum. In

the thalamus, Cx36 expression is absent or weak in most thalamic nuclei, with the exception of the reticular thalamic nucleus. Interestingly, this nucleus also contains GABAergic, parvalbumin-positive interneurons that are involved in genesis of thalamocortical oscillations (Pinault and Deschenes, 1992; Destexhe et al. 1996). Furthermore, Cx36 was detected in neurons of the centrolateral thalamic nucleus, which has been assigned to the intralaminar thalamocortical cell group, and these neurons are possibly involved in the distribution of coherent 40 Hz oscillations characterizing the magnetoencephalographic activity during wakefulness and REM sleep (Llinas and Ribary, 1993; Steriade et al. 1993). Functional analysis on Cx36-deficient brain tissue demonstrated that coupling of thalamic reticular neurons is almost entirely dependent on Cx36 (Landisman et al. 2002), and it was proposed that electrical synapses coordinate the rhythmic activity of small clusters of neurons within the thalamic reticular nucleus (Long et al. 2004).

In the striatum, parvalbumin-positive GABAergic neurons were shown to possess gap junctions (Kita et al. 1990). As this population comprises 3–5% of striatal neurons, the proportion is well in agreement with the low number of scattered cells expressing Cx36 mRNA (Condorelli et al. 2000). As interneurons receive information from the cerebral cortex, it is feasible that these Cx36-expressing neurons might participate in synchronization of striatal spiny neurons.

In the cerebellum, the subpopulation of parvalbumin-containing neurons of the molecular layer expresses Cx36 at detectable level. These inhibitory GABAergic interneurons, which are likely to correspond to stellate and/or basket neurons, display intense coupling including the generation of synchronous activity (Mann-Metzer and Yarom, 1999). In addition to its localization in interneurons of the cerebellar cortex, Cx36 mRNA was also detected in the deep cerebellar nuclei by *in situ* hybridization (Condorelli et al. 2000), and this data was confirmed in transgenic mice using a lacZ-Cx36 reporter gene (Degen et al. 2004). However, Cx36 protein could not be detected in GABAergic neurons of the cerebellar nuclei (Degen et al. 2004), a finding which was of particular interest in view of projections between cerebellar nuclei and the nuclear complex of the inferior olive: Cx36 protein was shown to be present in all nuclei of the inferior olive, but to be absent in neurons of the deep cerebellar nuclei. In contrast, the N36- β gal reporter protein was expressed in many but not all nuclei of the inferior olive and was present in all deep cerebellar nuclei (Degen et al. 2004). Although oscillations of olivary neurons still occur in Cx36-deficient mice (Long et al. 2002), the absence of electrical coupling nevertheless results in changes of olivary neuron oscillations, and also comprises gap junctional changes on the ultrastructural level in that the interneuronal space was abnormally wide (De Zeeuw et al. 2003).

Expression of Cx36 and the implication of electrotonic synapses in the network of **dopaminergic neurons** are hardly explored: Cx36 mRNA expression was detected in the substantia nigra (pars compacta and pars reticulata) and

the ventral tegmental area, with the cellular identity of Cx36-positive cells demonstrated by double labelling with tyrosine hydroxylase (Condorelli et al. 2000). However, the functional impact of Cx36 in the dopaminergic neuronal network has not been demonstrated yet, although there is electrophysiological evidence for electrotonic coupling of dopaminergic neurons (Freeman et al. 1985; Vandecasteele et al. 2005).

Connexin36 expression has also been described in various other neuronal cells, and shall be summarized briefly. In spinal cord, neurons of all laminae express Cx36 mRNA, with highest levels of expression being detected in **motoneurons** (Condorelli et al. 2000), however, gap junctional coupling amongst spinal motoneurons was only observed during early postnatal development (Fulton et al. 1991). In **hypohalamic neurons**, dye- and electrotonic coupling has been demonstrated in paraventricular and supraoptic nuclei (Andrew et al. 1981; Yuste et al. 1992; Micevych et al. 1996). Gap junctions in the **olfactory bulb** have already been demonstrated (Landis et al. 1974), and *mitral cells* were shown to contain Cx36 using immunohistochemistry and transgenic mice (Meier et al. 2002; Degen et al. 2004). When the role of gap junctions between mitral cells was investigated in Cx36-deficient mice, electrical coupling and correlated spiking between mitral cells was entirely absent (Christie et al. 2005).

6.1.4

Connexin Gap Junctions in the Retina

From the expression studies and functional analysis summarized above, it is tempting to postulate that gap junctions form communication compartments. The retina represents a two-dimensional stratified tissue of the brain with populations of well-characterized neurons (Haverkamp and Wassle, 2000). Because of its layered organization, the retina comprises a system in which cell interaction, electrical communication, and its ultrastructural correlate can straightforwardly be investigated.

Microinjection studies applying neurobiotin as a gap junction permeable tracer have led to the discovery of a high incidence of coupled cells and revealed a diversity of coupling patterns so far unmatched in any other part of the brain (Vaney, 1999). The predominant connexin in retinal neurons is Cx36. A number of interneurons, including AII amacrine cells (Feigenspan et al. 2001; Guldenagel et al. 2001; Mills et al. 2001), principle cells in the form of alpha ganglion cells (Schubert et al. 2005), cone photoreceptors (Lee et al. 2003; Feigenspan et al. 2004), as well as OFF cone bipolar cells (Feigenspan et al. 2004), have been described as expressing Cx36 (Fig. 3). Although Cx36 has previously been associated with the rod pathways of the retina (Mills et al. 2001; Deans et al. 2002; Lee et al. 2003), its expression in rods has been questioned in another study (Feigenspan et al. 2004). The pattern of retinal connexin expression has become more complex by the discovery of cell-

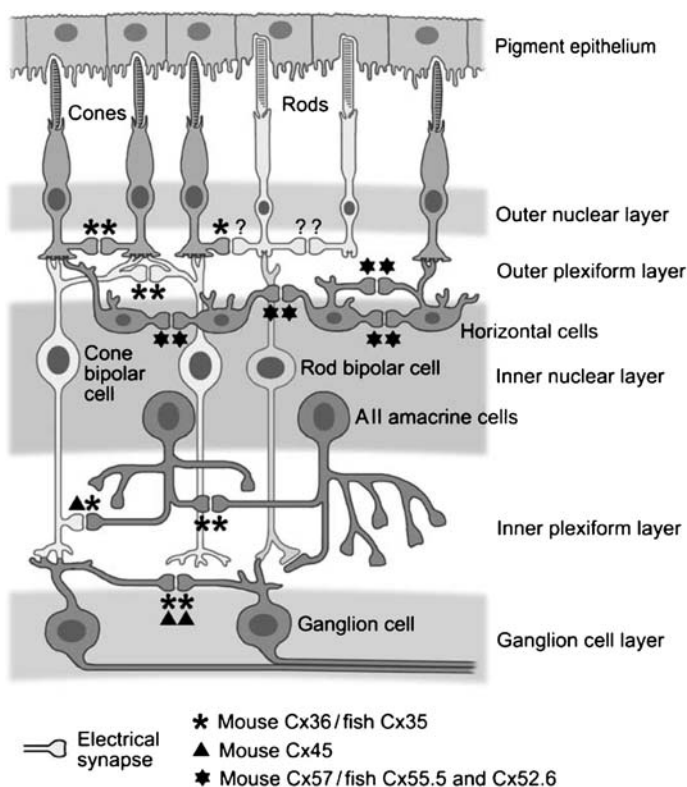


Fig. 3 Schematic representation of gap junctions in the retina. Neurons of the retina express several connexin proteins, including Cx36, Cx45 and Cx57 in the mouse, and Cx35, Cx55.5 and Cx52.6 in fish. Electrical synapses are formed between the neuronal subtypes of the retina

specific expression, which includes Cx45 (Guldenagel et al. 2000; Maxeiner et al. 2005). Using targeted deletion of Cx45 and concomitant activation of the enhanced green fluorescent protein (EGFP), EGFP labelling was observed in bipolar, amacrine and ganglion cell populations. Intracellular microinjection of fluorescent dyes in EGFP-labelled somata combined with immunohistochemical markers revealed Cx45 expression in both ON and OFF cone bipolar cells (Maxeiner et al. 2005). Functional analyses of both Cx36 and Cx45 deleted mice indicated that both connexins are involved in signal transmission of the major rod pathway (Deans et al. 2001; Maxeiner et al. 2005). The scotopic electroretinogram of mutant mice revealed a normal a-wave but a reduction in the b-wave amplitude (Maxeiner et al. 2005), similar to that found in Cx36-deficient animals (Guldenagel et al. 2001). Furthermore, neurotransmitter coupling between AII amacrine cells and Cx45-expressing cone bipolar cells was disrupted in Cx45-deficient mice. These data suggest that both Cx36 and Cx45 participate in the formation of functional heterotypic

electrical synapses between these two types of retinal neurons that make up the major cone pathway.

Studies on zebrafish retina indicated that additional connexins are involved in the formation of electrical synapses. Cloning of four zebrafish connexins (Dermietzel et al. 2000b; Zoidl et al. 2004) and evaluation of their cell-specific distribution showed that two of these connexins (zfCx55.5 and zfCx52.6) are retina-specific and exclusively expressed in horizontal cells. Furthermore, these connexins show essentially different electrophysiological properties under functional expression conditions. In recently generated transgenic mice, Cx57 has been described as showing an identical pattern with restriction to horizontal cells (Hombach et al. 2004) as discovered for the two zebrafish connexins. This site-restrictiveness is a further indication that neurons can be endowed with their specific connexin complement and that multiple connexins with the potential for compatible heterotypic channel formation are likely to shape the functional connectivity of retinal circuits. Obviously, the network behaviour of electrical synapses seems to depend largely on the type of connexins expressed in individual classes of neurons and their combinatorial configuration in terms of homotypic and/or heterotypic combinations.

A further degree of complexity can be achieved if one considers the susceptibility of individual connexins to phosphorylation through kinase activation. In the case of horizontal cell electrical coupling a clear link between dopamine and the PKA pathway has consistently been reported (Piccolino et al. 1984; Hampson et al. 1992; Weiler et al. 2000). Dopamine exerts its influence on the size of the receptor field by decoupling horizontal cells. This mechanism becomes most effective under light exposure when dopamine secretion increases. This example shows a clear interference of neurotransmitter activity and functional behaviour of an electrically coupled network.

Even more intriguing are data provided by Mills and Massey (Mills and Massey, 1995) who showed that an individual class of neurons can be furnished with electrical synapses of different functional phenotypes. AII amacrine cells of the retina are coupled homotypically with AII amacrine neurons, and heterotypically with cone bipolar cells. After injecting a single AII amacrine cell, Mills and Massey found that neurobiotin (relative molecular mass, 286 Dalton) passed easily through both types of gap junctions, but that biotin-X cadaverine (relative molecular mass, 442 Dalton) passed through AII/bipolar cell gap junctions poorly compared to AII/AII gap junctions. Thus, the AII/bipolar cell channel has a lower permeability to large molecules than does the AII/AII amacrine cell channel. More strikingly, the two pathways are also regulated differently. Dopamine and cyclic AMP agonists, known to diminish AII–AII coupling, did not change the relative labelling intensity after intracellular dye injection of AII to bipolar cells. However, nitric oxide and cGMP agonists selectively reduced labelling in bipolar cells relative to AII amacrine cells, perhaps by acting at the bipolar side of this

gap junction. This suggests that gating mechanisms of both types of electrical synapses are driven by different signalling pathways, which are apt to control the network switch between rod and cone pathways associated with light (Mills and Massey, 1995).

Further neurotransmitter effects have been described for serotonin in the somatosensory cortex of postnatal rats (Rorig and Sutor, 1996), suggesting extensive interactions between synaptic transmission and information flow through gap junctions. Obviously, the interaction between gap junctions and chemical synaptic transmission represents a powerful tool that helps to sculpture synaptic circuits in the developing brain as well as to increase the efficacy of synaptic transmission in adult networks (Roerig and Feller, 2000). In addition, gap junctional communication seems to be modulated not only by classical neurotransmitters, but also seems to include neuroactive gaseous substrates, i.e. the nitric oxide (NO)/cGMP system (see above). Decoupling of retinal horizontal cells occurs upon NO release and it was suggested that the effect of NO on the responsiveness of horizontal cells might be due to an action on green- and red-sensitive cones (Pottek et al. 1997). Apparently, the interaction of neuroactive transmitters and electrical synapses includes not only wiring transmission but also entails volume transmission as well.

6.2

Pannexin Gap Junctions

Most of the neuronal connexins were identified at sites that previously were shown to be electrically coupled, albeit in the absence of Cx36 expression, and can therefore account for electrical synapse activity in these regions (Sohl et al. 2004, 2005). However, with the identification of a novel family of gap junction proteins named pannexins (see below), some of these assignments might need to be reconsidered.

Connexin36 is one of the major players in the electrical coupling between hippocampal interneurons. Although deletion of Cx36 resulted in the disruption of γ -frequency network oscillations in vitro and in vivo (Hormuzdi et al. 2001; Maier et al. 2002; Buhl et al. 2003), high frequency oscillations in the hippocampus, which are likely to involve pyramidal cells (Schmitz et al. 2001), were unaffected (Hormuzdi et al. 2001). Explanation for the latter observation can be provided by either of two hypotheses: The gap junction protein in pyramidal cells causing high frequency oscillations might be (1) a connexin protein different from Cx36 or (2) a distinctly different class of proteins.

Theoretical support for the latter hypothesis initially arose from in silico analysis of the vertebrate genomes identifying orthologous DNA sequences in the human genome that show similarity to the family of innexin proteins (i.e. “*invertebrate analogue of connexins*”) (Phelan et al. 1998; Phelan and Starich, 2001). Innexins were suggested to be specific invertebrate gap junction proteins, representing the functional correlate of connexin proteins in

invertebrates (Phelan et al. 1998; Phelan and Starich, 2001; Hua et al. 2003), i.e. forming gap junction communication channels (Phelan et al. 1998; Landesman et al. 1999; Stebbings et al. 2000). Although unrelated to connexins at the sequence level, innexins also share certain topological features with connexin proteins, including the presence of four transmembrane domains (Bruzzone et al. 1996; Phelan and Starich, 2001).

With the cloning of innexin homologues in vertebrates, these proteins had to be reclassified, and innexins were subsequently incorporated in the group of pannexin proteins (latin: pan – all, throughout; nexus – connection, bond), a name indicating the ubiquitous distribution of this protein family in invertebrates as well as chordates (Panchin et al. 2000). Meanwhile, three pannexin-encoding genes have been cloned from the human and mouse genomes, respectively, and named PANX1 (Panx1), PANX2 (Panx2) and PANX3 (Panx3) (Panchin et al. 2000; Bruzzone et al. 2003; Baranova et al. 2004; Dykes et al. 2004). Expression analysis on mRNA level has revealed a distinct distribution of these three genes in developing and adult animals (Bruzzone et al. 2003; Baranova et al. 2004) with Panx1 and Panx2 being co-expressed in a number of tissues. Pannexin-1 and Panx2 mRNAs are both present in the central nervous system, including cortex, striatum, olfactory bulb, hippocampus, thalamus, cerebellum and inferior olive (Bruzzone et al. 2003; Ray et al. 2005; Weickert et al. 2005). In contrast, expression of Panx3 seems to be absent from the adult rat nervous system with its expression sites being in skin, osteoblasts and synovial fibroblasts (Panchin et al. 2000; Bruzzone et al. 2003; Baranova et al. 2004). However, RT-PCR analysis from human adult hippocampus samples revealed an amplification product (Baranova et al. 2004). Another apparent difference between species was observed in the expression of Panx2, which appears to be absent from spinal cord in human tissue (Baranova et al. 2004), whereas in mouse, Panx2 expression was shown to be higher than that of Panx1 (Bruzzone et al. 2003).

The presence of pannexin genes in the nervous system points to a possible function of the gene product in neuronal communication. This implication is supported by the notion that Panx1 and Panx2 mRNAs are expressed in hippocampal pyramidal cells (Bruzzone et al. 2003; Ray et al. 2005; Weickert et al. 2005), which were previously characterized by the near-absence of Cx36, but are nevertheless dye-coupled (MacVicar and Dudek, 1980; MacVicar et al. 1982), and electrically coupled (MacVicar and Dudek, 1981; MacVicar and Jahnsen, 1985). Direct proof for the functional implication of pannexin channels was provided by Bruzzone and co-workers (Bruzzone et al. 2003) who demonstrated in paired *Xenopus* oocytes that rodent Panx1 – alone or in combination with Panx2 – formed intercellular channels. Heteromeric Panx1/Panx2 channels, albeit functional, displayed a reduction in current amplitude, and a slower gating mechanism. Neither Panx2 nor Panx3 induced membrane currents above those recorded from controls. Intriguingly, there is pharmacological evidence that pannexin channels respond to some

inhibitors known to block gap junction channels composed by connexin proteins, whereas others proved ineffective (Bruzzone et al. 2005). Pannexin-1 expressed in single *Xenopus* oocytes was shown to form functional hemichannels (Bruzzone et al. 2003), which is of particular interest in view of functional evidence demonstrating the mechanosensitivity of pannexin channels causing the release of ATP to the extracellular space upon mechanical stress (Bao et al. 2004).

Taken together, the expression patterns and electrophysiological properties of Panx1 and Panx2 provide evidence that pannexins represent a second family of gap junction proteins in vertebrates. However, functionality in the CNS still needs to be determined, and studies on pannexin-deficient mice will need to demonstrate the functional impact of pannexin channels on high-frequency oscillations and neuronal network synchronization. To understand the channel-forming properties of pannexins, elucidation of their ultrastructure is also essential.

7

Conclusions

The impact of electrical synapses in the mammalian nervous system has been recognized over the past few years, and electrotonic coupling has been accepted as a major player in neuronal interaction. One major breakthrough was achieved with the cloning of Cx36, as identification of a gap junction protein predominantly expressed in neurons led to the detailed analysis of neuronal networks and the impact of electrical synapses in their synchronization. Connexin36-deficient mice allowed the possibility of investigating neuronal interaction in the absence of electrical Cx36 synapses. These studies revealed a major involvement of Cx36 in synchronizing neuronal network oscillations in various neuronal systems, for instance in the synchronization of γ -frequencies in GABAergic interneurons of the hippocampus. With the identification of the family of pannexin gap junction proteins, the studies will have to be extended to the analysis of the influence of pannexins on electrical communication between neurons, a task that will certainly be addressed upon generation of pannexin-deficient animals. With these important studies, it will also be able to resolve the question of whether connexin and pannexin gap junction channels function separately or are somewhat orchestrated.

The second major topic, which arose with the identification of the involvement of electrical synapses in neuronal networks, is the functional and structural interaction of chemical and electrical transmission. In several studies, chemical synapses were shown to influence electrical coupling and vice versa (Montoro and Yuste, 2004). Examples of neurotransmitter action of electrical synapses include the effects of dopamine, in that extensive coupling

of horizontal cells in the turtle retina was greatly reduced by application of dopamine (Dowling, 1991), and dye communication between cortical neurons of the neonatal rat largely disappeared upon dopamine application to brain slices (Rorig et al. 1995).

Further implications for a possible interaction of electrical and chemical synapses were provided by ultrastructural evidence for the presence of so-called mixed synapses. The system in which these interactions were studied most extensively is the Mauthner cell of the goldfish (Pereda et al. 1994); electrical transmission at club endings of these cells was already described in 1962 (Furshpan and Furukawa, 1962). More recently, these electrical synapses have been visualized by the FRIL technique, and their connexin components identified as being Cx35, the fish orthologue of mammalian Cx36 (Pereda et al. 2004; Rash et al. 2004). Interestingly, these gap junction plaques were found in close vicinity to neurotransmitter receptors, i.e. the NMDA-receptor 1 subunit of glutamate receptors (NR1), pointing to the interaction of chemical and electrical transmission. Mixed synapses have meanwhile also been identified in neurons of the nuclear inferior olivary complex (Rash et al. 2004), and it seems likely that the occurrence of mixed synapses will not be restricted to these sites.

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Neuron-Glia Interactions at the Node of Ranvier

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Abstract Rapid, faithful, and efficient action potential propagation in mammalian axons is a consequence of myelin and clustered Na⁺ channels. Both myelination and node of Ranvier formation require complex intercellular interactions between neurons and glia that result in profound molecular, morphological, and functional changes in each cell type. This review will focus on the molecular and cellular mechanisms that underlie neuron-glia interactions at the node of Ranvier. In particular, the proteins and protein complexes, and how they participate in node of Ranvier formation and maintenance, will be discussed. Traditionally, myelinating glia have been viewed as merely passive players in neuronal function, conferring on the axons they ensheath various electrical properties that facilitate action potential conduction. However, it is now recognized that this view is incomplete. This review will discuss several examples illustrating how myelinating glia actively regulate the excitable properties of axons including the kinds of channels expressed and their subcellular localization.

1

Introduction

Intercellular communication is essential for proper nervous system function. The prototypical example of this interaction is found at the synapse. Here, pre- and postsynaptic neurons form functionally, morphologically, biochemically, and molecularly distinct structures that permit rapid, faithful and efficient transmission of information. However, before synaptic transmission can ever take place, action potentials must be conducted along axons that traverse relatively long distances. As with the synapse, axons must also facilitate rapid, faithful, and efficient action potential propagation while minimizing both the metabolic and space requirements needed to accomplish this. Vertebrates have solved these problems through the development of 1) the myelin sheath (Fig. 1A) and 2) the clustering of voltage-gated Na⁺ (Nav) channels at regularly spaced gaps in the myelin sheath called nodes of Ranvier (Fig. 1B,C). Like the synapse, the myelin sheath and the node of Ranvier are functionally, morphologically, biochemically, and molecularly distinct structures.

Myelin is a multilamellar structure that ensheathes axons and is composed of both lipid and protein. Myelin is made in the central nervous system (CNS) by oligodendrocytes and in the peripheral nervous system (PNS) by

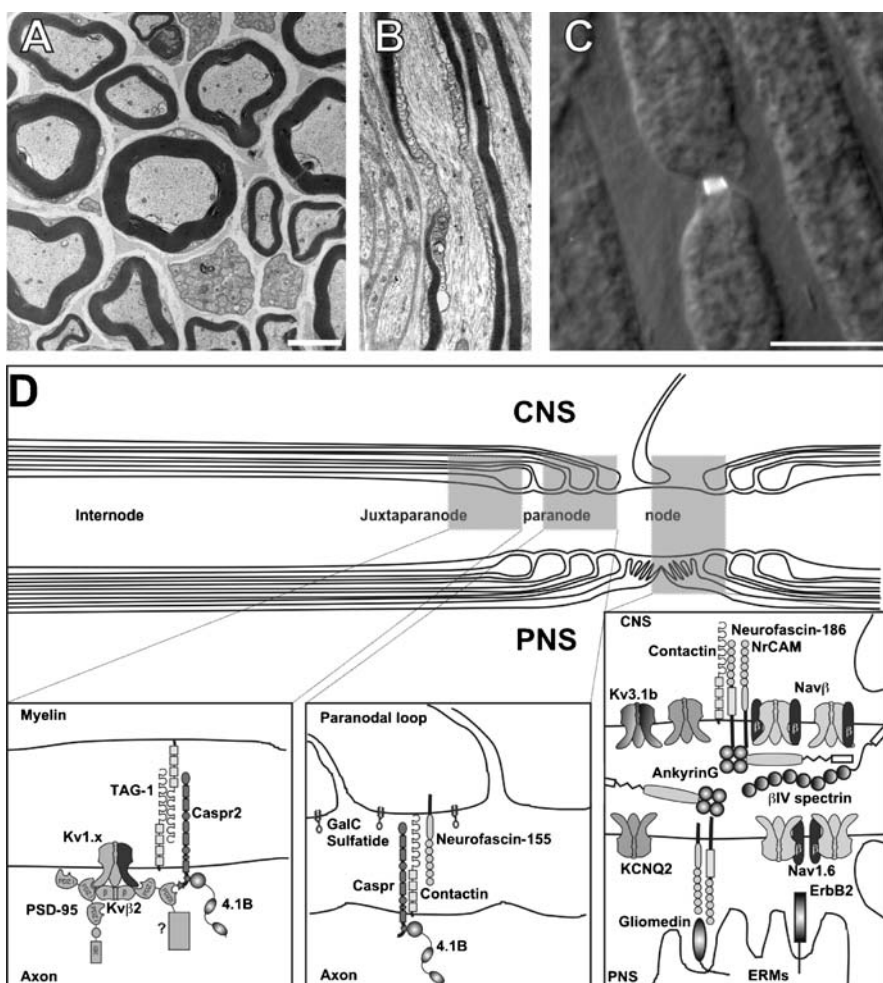


Fig. 1 Myelinated axons have several subdomains: nodes, paranodes, juxtaparanodes, and internodes. **A** Electron micrograph and cross-section of peripheral nerve showing myelinated axons. **B** Electron micrograph and cross-section of a node of Ranvier. **C** Peripheral node of Ranvier immunofluorescently labeled using antibodies against Nav channels. The outline of the myelin is visible using phase-contrast imaging. **D** Cartoon model illustrating many of the proteins found at nodes, paranodes, and juxtaparanodes (for details see text). Electron micrographs were kindly provided by Yang Yang. Scale bars: A = 2 μ m; C = 5 μ m

Schwann cells. Traditionally, the myelin membrane has been viewed as a passive contributor to action potential conduction: myelin increases membrane resistance and decreases membrane capacitance, thereby conserving ionic charge as the axolemma is depolarized during action potential propagation. However, this myopic view of myelin as simply a passive cellular structure is now recognized as incomplete, since oligodendrocytes and Schwann cells

actively regulate many neuronal properties, including *axonal* ion channel expression and localization.

Although the process of myelination itself provides an excellent example of reciprocal subcellular differentiation between neurons and glia (Michailov et al. 2004), the node of Ranvier is a particularly elegant and beautiful example. Furthermore, recent work has led to a dramatic increase in our knowledge of the molecules and mechanisms regulating node of Ranvier formation. This chapter will focus first on the proteins present at the node of Ranvier (and its associated domains, see Section 2.1), and then describe experiments examining some of these molecules (e.g. their function, localization, and/or redistribution under developmental or pathological conditions) that illustrate how the node of Ranvier is a prime example of neuron-glia interactions.

2

Domain Structure of the Myelinated Axon

Myelinated axons can be subdivided into several distinct domains: node of Ranvier, paranode, juxtaparanode, and internode (Fig. 1D). Each domain is characterized by a unique set of ion channels, cell adhesion molecules, scaffolding proteins, and cytoskeletal components.

2.1

Node of Ranvier

Morphologically, the node of Ranvier appears as a gap in the myelin sheath. A number of other structural features have been identified that are typical of nodes of Ranvier in both the CNS and PNS (see Peters et al. 1976). For example, electron microscopy of nodes reveals an electron dense cytoplasmic undercoating of the axolemma, and flanking paranodal “loops” of membrane bound cytoplasm (Fig. 1B). In general most features of CNS nodes are also found at PNS nodes. However, some differences do exist and these will be emphasized below.

Not surprisingly, nodes of Ranvier are molecularly distinct structures. Functionally, the most important and significant component of the node is the Nav channel. The main Nav channel subunit expressed at nodes of Ranvier is Nav1.6 (Fig. 2A; Caldwell et al. 2000). However, during early development, Nav1.2 is also found at nodes, and in the CNS precedes detection of Nav1.6 (Boiko et al. 2001). Thus, there is some developmental regulation of Nav channel subtype (for more details, see Section 3.1). Nav channels are often associated with accessory β -subunits, which can modulate the properties of Nav channels and promote their surface expression (Isom 2001). Nav β 1 is detected at nodes of Ranvier, and has been suggested to be important for

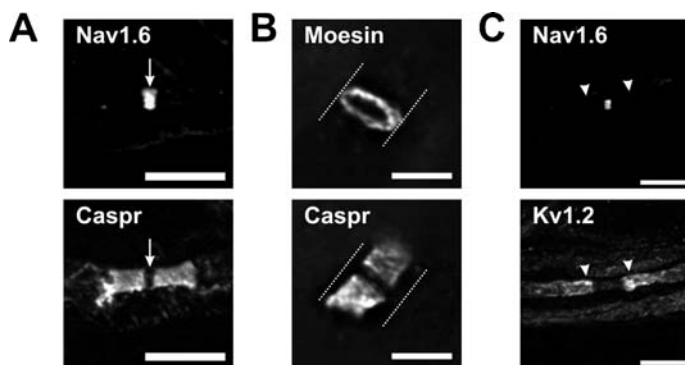


Fig. 2 Components of nodes, paranodes, juxtaparanodes, and Schwann cell microvilli. **A** Nodal Nav1.6 (*top*; *arrow*) and paranodal Caspr (*bottom*). **B** Paranodal Caspr (*bottom*) and Moesin found in Schwann cell microvilli (*top*); the *dotted lines* show the outermost edges of the Schwann cell microvilli staining. **C** Nodal Nav1.6 (*top*) and juxtaparanodal Kv1.2 (*bottom*; *arrowheads*). Scale bars = 5 μ m

neuron-glia interactions as well as regulating the fastest conducting axons, since these features are both disrupted in Nav β 1-null mice (Chen et al. 2004). Besides the accessory Nav β -subunits, the cytoplasmic protein FGF homologous factor 2B (FHF2B) has also been shown to associate with Nav1.6 at nodes of Ranvier (Wittmack et al. 2004). Surprisingly, FHF2B is only found at nodes of Ranvier in peripheral sensory axons. FHF2B does modify the biophysical properties of Nav1.6, and the changes may account for the subtle differences in the sodium currents reported in sensory versus motor axons (Wittmack et al. 2004).

The molecular identities of two nodal K⁺ channels were discovered by Devaux et al. (2003, 2004). These channels include KCNQ2 (also known as Kv7.2) and Kv3.1b; surprisingly, the latter is detected only in a subset of CNS nodes. The precise function of Kv3.1b is unknown, since mice deficient for these subunits show no change in conduction properties; it is possible that some other Kv3.X subunit compensates for the loss of Kv3.1b. In contrast, KCNQ2 channels appear to be functional at nodes of Ranvier, since application of the KCNQ2 agonist retigabine reduces axonal excitability (Devaux et al. 2004).

Several different cell adhesion molecules (CAMs) have also been described at nodes of Ranvier, including two members of the L1 family of CAMs (Nr-CAM and Neurofascin-186; Davis et al. 1996). However, the functions of these proteins are not well understood. Based on their localization during early developmental myelination, it has been suggested that they act as pioneer molecules, serving as nucleation sites for recruitment of cytoplasmic scaffolding proteins such as AnkyrinG, since these CAMs have a conserved cytoplasmic AnkyrinG binding domain (Lambert et al. 1997; Jenkins et al. 2001). The extracellular domains of these proteins may interact with glial CAMs to direct their localization along the axon (Poliak and Peles 2003); in-

deed, this function for nodal CAMs has now been verified in the PNS (see below, and Eshed et al. 2005). Interestingly, NrCAM-null mice have nodes of Ranvier, but Nav channel and AnkyrinG clustering is delayed compared with control mice (Custer et al. 2003). It is likely that Neurofascin-186 compensates for the loss of NrCAM, but with a short delay. The GPI-anchored CAM Contactin has also been shown to be present at nodes of Ranvier in the CNS (Rios et al. 2000), where it may participate in neuron-glia interactions and/or modulate the surface expression of Nav channels (Kazarinova-Noyes et al. 2001). Finally, Nav β 1 has several properties of a CAM and has been shown to be able to interact with the other CAMs described above (McEwen and Isom 2004). The significance of these interactions is unknown, but may be reflected in the subtle structural paranodal/nodal abnormalities observed in the Nav β 1-null mouse (Chen et al. 2004).

The scaffolding proteins AnkyrinG and β IV spectrin are also found in high densities at nodes of Ranvier. AnkyrinG has been shown to interact with β IV spectrin, Nav channels, and the CAMs Neurofascin-186 and NrCAM (Tuvia et al. 1997; Komada and Soriano 2002; Garrido et al. 2003a). As such, AnkyrinG may act as the main scaffold, linking transmembrane proteins to the actin-spectrin based cytoskeleton. Consistent with this idea, mice with a cerebellum specific deletion in AnkyrinG show abnormal clustering of Nav channels and Neurofascin-186 at axon initial segments, a membrane domain that in many ways (functionally, molecularly, and ultrastructurally) is similar to the node of Ranvier (Zhou et al. 1998; Ango et al. 2004). However, mutations in β IV spectrin have also been shown to compromise nodal membrane structure and lead to lower densities of Nav channels and AnkyrinG (Komada and Soriano 2002; Yang et al. 2004). Thus, future experiments will be needed to determine whether AnkyrinG recruits β IV spectrin (and other components) to nodes of Ranvier and axon initial segments, or vice versa.

One major difference between nodes of Ranvier in the PNS and CNS is that the former is contacted by numerous finger-like protrusions from the Schwann cells called microvilli (Fig. 1D). The microvilli have been shown to contain a variety of signaling, adhesion, and structural proteins, including ERMs (Ezrin, Radixin, and Moesin), EBP50 (Ezrin binding protein 50 kD), erbB2, Syndecans, NSF, Rho, and Gliomedin (Fig. 2B; Melendez-Vasquez et al. 2001, 2004; Scherer et al. 2001; Goutebroze et al. 2003; Taylor et al. 2004; Eshed 2005; Guertin et al. 2005). Interestingly, many of these microvilli proteins accumulate at the tips of myelinating Schwann cells during early development (Melendez-Vasquez et al. 2004). Some experiments have even suggested that these microvilli may be important contributors to Nav channel clustering (Gatto et al. 2003). The observation that mutant animals with aberrant Schwann cell microvilli also have disrupted Nav channel clusters is consistent with a role for microvilli in both forming and maintaining nodes (Saito et al. 2003; Yang et al. 2004). In fact, this role for microvilli has now been clearly established with the identification of Gliomedin, a cell-adhesion molecule

found at the tips of the Schwann cell microvilli, as the Schwann cell receptor for nodal NF-186 (Eshed et al. 2005). Indeed, it now appears that in the PNS Gliomedin directs the initial events surrounding node of Ranvier formation (see below). Although CNS nodes do not have microvilli, some nodes appear to be partially enveloped by a process from astrocytes or NG2+ glial cells that have recently been termed “synantocytes” (Butt et al. 2002). These perinodal processes may function in a manner similar to Schwann cell microvilli, or they have also been proposed to be involved in buffering ions from the perinodal space; future experiments will need to test these possibilities.

Other molecules in the extracellular space may contribute to CNS node formation, maintenance, and/or function. For example, the molecules tenascin (matrix molecule) and Oligodendrocyte Myelin Glycoprotein (OMGp; GPI anchored CAM) are detected at CNS nodes of Ranvier (Bjartmar et al. 1994; OMGp, M.N. Rasband, unpublished results). The function(s) of these proteins are unknown. Interestingly, mice lacking Tenascin-R show a significant reduction in compound action potential conduction velocity, although both nodes of Ranvier and myelin appear normal (Weber et al. 1999). OMGp has recently been shown to bind to the Nogo Receptor and inhibit neurite outgrowth (Wang et al. 2002). Given this function, it is easy to speculate that OMGp may participate in limiting collateral sprouting from nodes of Ranvier.

2.2

Paranode

The paranodes flank nodes of Ranvier and are the largest vertebrate junctional adhesion complex (Peters et al. 1976; Rosenbluth 1995). Although in longitudinal sections the paranodal loops appear as independent entities (Fig. 1B), in fact they are a continuous helical structure that winds around the axon. Paranodal loops appose the axon very closely and beneath each loop of cytoplasm a set of electron dense bands (called transverse bands) can be detected. Structurally, these junctions are very similar to the *Drosophila* salivary epithelial septate junctions. The septate junctions function to provide a paracellular diffusion barrier, a role that is analogous to that performed by the paranodal loops in myelin. Moreover, the protein components of paranodes share homology with invertebrate septate junction proteins. Important insights into the formation and function of paranodes have come from analysis of *Drosophila* mutants with disrupted septate junctions. For example, deletion of the protein neurexin leads to septate junction breakdown and loss of the diffusion barrier. The mammalian homologue to neurexin is the Contactin Associated Protein (Caspr; also known as Paranodin or NCP1; Einheber et al. 1997; Menegoz et al. 1997; Peles et al. 1997; Bhat et al. 2001). Caspr is an axonal transmembrane protein that is found at very high densities at paranodes (Fig. 2A,B). Interestingly, Caspr-null mice lack transverse bands, have disrupted nodal domain structure, show lateral diffusion of nodal ion channels

into paranodes, and have a reduced compound action potential conduction velocity (Bhat et al. 2001).

As its name suggests, Caspr interacts in *cis* with contactin, a GPI anchored cell adhesion molecule (Peles et al. 1997); *Drosophila* contactin is also found at epithelial septate junctions (Faivre-Sarrailh et al. 2004). Contactin is required for the cell-surface expression of Caspr (Faivre-Sarrailh et al. 2000). Furthermore, mice lacking contactin show disrupted nodal structure and have a very similar phenotype to that observed in the Caspr-null mouse (Boyle et al. 2001). Another component of *Drosophila* septate junctions is neuroglian (NRG). This protein is a binding partner for neurexin and flies with a mutant form of NRG have disrupted septae (Genova and Fehon 2003). Remarkably, the mammalian homologue to NRG is the CAM Neurofascin, and a glial specific splice variant, Neurofascin-155 (NF-155; recall that the neuron specific splice variant, NF-186, is found at high densities at nodes of Ranvier), has been localized to the paranode (Tait et al. 2000). While some evidence suggests that Caspr, contactin, and NF-155 form a tripartite protein complex (Charles et al. 2002), other data argue that heterophilic interactions between only Contactin and NF-155 underlie the paranodal structure (Gollan et al. 2002). Whatever the specifics of the protein complex, the importance of both Contactin and Caspr is underscored by the observation that mice deficient for either one of these proteins lack appropriate paranodal junctions and have disrupted ion channel localization (Bhat et al. 2001; Boyle et al. 2001).

Integral membrane proteins are often restricted to specific membrane domains through interactions with cytoskeletal scaffolding or adaptor proteins. Caspr has now been shown to colocalize and interact with protein 4.1B (Ohara et al. 2000; Denisenko-Nehrbass et al. 2003). The 4.1 family of proteins links membrane proteins to the actin-spectrin based cytoskeleton. During development, protein 4.1B appears at paranodes after Caspr, suggesting that the primary role of protein 4.1B is to anchor and maintain the paranode, rather than precipitate clustering of Caspr and formation of the paranode (Denisenko-Nehrbass et al. 2003). It will be interesting to determine whether specific spectrins and/or actins are restricted to the paranode, in a manner similar to the restricted localization of β IV spectrin to nodes of Ranvier.

2.3

Juxtaparanode

The juxtaparanode begins at the innermost axoglial junction of the paranode. This domain extends into the internode for 5–15 μ m, and is characterized by high densities of voltage-gated Kv1 channels (Fig. 2C; Chiu and Ritchie 1980; Wang et al. 1993). Kv1 channels are composed of a tetramer of pore-forming, voltage-sensitive α -subunits, and up to four cytoplasmic β -subunits that have been shown to promote channel surface expression (Manganas and Trim-

mer 2000). Kv1 channel α -subunits can form heterotetrameric channels, but only a relatively small number of subunit combinations have been detected (Rhodes et al. 1995; Shamotienko et al. 1997; Rasband and Trimmer 2001; Rasband et al. 2001). Based on colocalization and co-immunoprecipitation experiments, juxtaparanodal Kv1 channel complexes are thought to consist of Kv1.1/Kv1.2/Kv β 2, Kv1.1/Kv1.4/Kv β 2, or Kv1.1/Kv1.2/Kv1.4/Kv β 2 heteromultimers (Wang et al. 1993; Rasband and Trimmer 2001; Rasband et al. 2001). It is clear that some axons contain one repertoire of channel subunits, while another contains a different set. The reasons for this are unknown. Since Kv1 channels are restricted to juxtaparanodes, it is reasonable to assume that these channels play important roles in nervous system function. However, the only clear evidence for a role in regulating action potential conduction is seen during early development when these Kv1 channels stabilize nodal excitability (Vabnick et al. 1999). Thus, the normal function of these channels remains an enigma (for a more complete description of the potential role(s) of juxtaparanodal Kv1 channels, see Chiu et al. 1999; Rasband 2004).

As at the node of Ranvier and the paranode, a larger protein complex exists at the juxtaparanode that has been shown to be important in regulating the localization of Kv1 channels (see Section 3.3). Further, like the node and paranode, there are both CAMs and scaffolding proteins that link to the Kv1 channel. Another member of the Caspr family of proteins, Caspr2, was shown to bind indirectly to Kv1 channels through a PDZ-domain protein (Poliak et al. 1999). Surprisingly, although the PDZ protein PSD-95 is present at juxtaparanodes and interacts with Kv1 channels, mice lacking juxtaparanodal PSD-95 have no apparent defects in Kv1 channel localization (Rasband et al. 2002). Thus, another yet to be identified scaffolding protein must link Kv1 channels and Caspr2.

Similar to its close homologue Caspr, Caspr2 also interacts with a GPI-anchored CAM, Transient Axonal Glycoprotein or TAG-1 (Traka et al. 2002; recall that Caspr interacts with the GPI-anchored protein Contactin), and protein 4.1B (Caspr2 has a 4.1B binding domain similar to Caspr; Denisenko-Nehrbass et al. 2003). Interestingly, TAG-1 is found in *both* the axonal and glial juxtaparanodal membranes (Traka et al. 2002; Poliak et al. 2003); however, other functions unrelated to myelinated nerve fibers have been suggested for this protein (Furley et al. 1990). The functions of TAG-1 and Caspr2 will be discussed in detail in Section 3.3.

3

Examples of Neuron-Glia Interactions

As described above, nodes, paranodes, and juxtaparanodes each have their own set of defining proteins. This section will summarize experiments show-

ing that neuron-glia interactions are essential for the formation and maintenance of these domains.

3.1

Neuron-Glia Interactions Regulate Nav Channel Clustering at Nodes of Ranvier

High-density clusters of Nav channels are the hallmark of nodes of Ranvier. As described above, these $\sim 1 \mu\text{m}$ long clusters are found at regularly spaced gaps in the myelin sheath along the entire length of axons, which in humans can be 1 m or more in length. Thus, a single nodal cluster of Nav channels occupies approximately 1/1000 000th of the axonal membrane. How is this precise targeting possible? Clearly, some exquisite neurobiological mechanisms must exist to establish and maintain these precisely defined domains. Does the neuron specify and maintain the nodal Nav channel cluster through intrinsic determinants and mechanisms, or do extrinsic factors contributed by glia influence Nav channel clustering? Or is there a combination of intrinsic and extrinsic factors that work together to establish and maintain Nav channel clusters at nodes of Ranvier?

Some of the first experiments to address the mechanism(s) of Nav channel clustering focused on remyelination and developmental myelination in the PNS (Dugandzija-Novakovic et al. 1995; Vabnick et al. 1996). An important property of Schwann cells is that after injury or disease, they can fully remyelinate demyelinated axons. However, the process of remyelination results in the formation of much shorter myelin segments. Thus, many new nodes of Ranvier must be established and maintained to allow for normal action potential conduction through the remyelinated zone. Dugandzija-Novakovic et al. (1995) induced acute and focal demyelination in sciatic nerve axons using a single intraneural injection of the detergent lysolecithin, then carefully determined the localization of Nav channels after demyelination, and during the process of remyelination. Similarly, Vabnick et al. (1996) characterized the localization of Nav channels during developmental myelination. Both studies showed that as Schwann cells begin to form myelin sheaths, Nav channels accumulate at the edges of the newly forming sheath and are “pushed” towards adjacent Schwann cells. Finally, these Nav channel clusters flanking each sheath fuse into new nodes of Ranvier. Importantly, in both studies, when myelination was inhibited, by blocking Schwann cell proliferation, Nav channel clusters failed to form.

Consistent with these *in vivo* observations, Ching et al. (1999) showed that in an *in vitro* myelinating model Nav channels were efficiently clustered and formed nodes of Ranvier in dorsal root ganglion axons, but only when Schwann cells were in contact with the axon and allowed to form myelin. Taken together, these early experiments suggested a model for PNS node formation where extrinsic factors (i.e. contact with myelinating Schwann cells) mediate Nav channel clustering. This long sought for contact-dependent, ex-

trinsic factor has now been identified as a Schwann cell microvilli protein called Gliomedin (Eshed et al. 2005). During development Gliomedin binds directly to axonal NF-186 and NrCAM. These CAMs then begin to accumulate at the leading edges of myelinating Schwann cells (where the microvilli are located). NF-186 then recruits AnkyrinG to newly forming nodes of Ranvier. Finally, AnkyrinG acts as a scaffold for the binding and localization of other nodal proteins such as Nav channels (Garrido et al. 2003b). It is not clear yet whether AnkyrinG brings Nav channels with it from the cell body, or whether it is clustered first and Nav channels arrive later. Some data suggest that AnkyrinG is detected at new nodes before Nav channels are clustered (Rasband et al. 1999). While this model is very concise and simple, it may be that specific recruitment of Nav channels by Gliomedin and exclusion of Nav channels from sites of close axoglial contact (i.e. paranodes) both contribute to the restricted localization of Nav channels at nodes of Ranvier. Future studies examining mice lacking Gliomedin will no doubt help to establish whether paranodal axoglial contact also contributes to Nav channel clustering.

Is contact with myelinating oligodendrocytes necessary for Nav channel clustering in the CNS? Since nearly every molecule found at nodes, paranodes, and juxtaparanodes in the CNS is also found at nodes in the PNS (with the important exception of the microvilli proteins and especially Gliomedin; Eshed et al. 2005), it is not difficult to imagine that CNS node of Ranvier formation requires myelinating oligodendrocytes, just as PNS node formation requires myelinating Schwann cells. Early *in vitro* studies showed that the number of Nav channel clusters dramatically increases in retinal ganglion cell axons when cells are grown in oligodendrocyte conditioned medium; astrocyte conditioned medium does not have a similar effect (Kaplan et al. 1997). Furthermore, the spacing between these individual clusters is consistent with internodal distance *in vivo*. While these results are consistent with oligodendrocytes providing an extrinsic factor that initiates Nav channel clustering, an important difference is that the results of Kaplan et al. (1997) argue that contact is not an essential component of Nav channel clustering in the CNS.

The identification of components of the axoglial junction allowed for a more direct assessment of the role of neuron-glia contact in CNS node formation. Rasband et al. (1999a) used a combination of electrophysiology and immunofluorescence microscopy to examine the events surrounding node of Ranvier formation and axonal function in optic nerve axons. Specifically, antibodies against Caspr and Nav channels were used to determine when paranodes began to form and when Nav channel clusters first appeared. Interestingly, paranodal axoglial junctions, defined by Caspr immunoreactivity, were detected prior to the accumulation of any Nav channel clusters. Early Nav channel clusters were often elongated and were always immediately adjacent to Caspr-labeled axoglial junctions, without any overlap in Caspr and Nav channel immunoreactivity. As myelination progressed, and adjacent axoglial junctions approached one another, the Nav channel clusters became

much more focal. The patterns of immunoreactivity during node formation included heminodes (single channel cluster adjacent to axoglial junction), binary clusters (doublet of Nav channel clusters flanked on one side with Caspr staining), and focal Nav channel clusters (flanked by Caspr on each side); these three patterns of Nav channel immunoreactivity are very reminiscent of the patterns seen during PNS myelination both *in vivo* and *in vitro* (Vabnick et al. 1996; Ching et al. 1999). During this same period of active node formation and myelination, action potential conduction velocity increased significantly. Rasband et al. (1999) quantified the appearance of Caspr-labeled paranodal axoglial junctions from postnatal day 5 through postnatal day 60 and found that these sites preceded the appearance of Nav channel clusters by about 2 days. Taken together, the morphology, timing, and close apposition of Caspr and Nav channel localization strongly suggest that *in vivo* Nav channels cluster only after oligodendrocytes form axoglial junctions and initiate myelination.

In support of the idea that myelination and neuron-glia interactions are important for Nav channel clustering in the CNS, Boiko et al. (2001) examined two different Nav channel subtypes, Nav1.2 and Nav1.6 in optic nerve axons. Importantly, Nav1.2 had been previously described in unmyelinated axons in the CNS (Gong et al. 1999), whereas Nav1.6 had been reported at nodes of Ranvier and axon initial segments (Caldwell et al. 2000). Given this difference in distribution, these authors wondered if myelination was in some way responsible for the differential expression of the two Nav channel subtypes. The choice of the optic nerve as a model was very important, since the anatomy of the optic nerve is such that as axons leave the retina and pass through the optic nerve head they are unmyelinated and remain unmyelinated until they pass through the lamina cribrosa, which is some distance from the optic nerve head. Thus, retinal ganglion cell axons have both a myelinated and an unmyelinated segment. Using antibodies against Nav1.2 and Nav1.6, Boiko et al. (2001) found that in the very same axon, Nav1.2 was found exclusively in the unmyelinated zone, whereas Nav1.6 was found only at nodes of Ranvier. The simplest explanation was that myelination directly regulates the kinds of channels found in the membrane, allowing Nav1.6 to be inserted at nodes of Ranvier, but excluding Nav1.2 from nodes and regions covered with myelin membrane. The details of this regulation are unknown.

To test directly whether myelin influences the kinds of channels expressed in axons, Boiko et al. (2001) determined the types of Nav channels expressed in optic nerve axons during early development. Initially, optic nerve axons are unmyelinated, yet are electrically active due to low densities of uniformly distributed Nav channels (Rasband et al. 1999a). What kinds of Nav channels are found in these immature optic nerve axons that only days later become myelinated? Interestingly, the earliest Nav channel clusters are composed of Nav1.2 subunits, consistent with the localization of Nav1.2 to unmyelinated axons in other regions of the CNS. These results suggest that in the CNS nodal

Nav channel clusters form from a pre-existing pool of Nav1.2 found in axons. During the next 2 weeks of most active myelination and node of Ranvier formation, Nav1.6 channels gradually replaced Nav1.2 channels at nodes of Ranvier. Thus, one interpretation is that myelination caused a switch in the kinds of Nav channels expressed at nodes of Ranvier.

The importance of myelin and axoglial contact in regulating the kinds of Nav channels expressed at nodes of Ranvier has been more directly verified in a variety of experimental models. For example, in the hypomyelinating mutant mouse *Shiverer*, compact myelin and axoglial junction formation are perturbed. As a consequence, Nav1.2 expression levels are dramatically increased (Westenbroek et al. 1992), and Nav channels fail to cluster appropriately (Rasband et al. 1999a; Boiko et al. 2001). When clusters do appear, they are almost invariably adjacent to some form of axoglial contact (reminiscent of paranodal axoglial junctions), and they almost always consist of Nav1.2 subunits.

One potential complication that arises from the *Shiverer* mice is that these animals have developmental hypomyelination, making it difficult to conclude that myelin actively regulates the kinds of channels expressed in axons. To address this more directly, two models of adult-onset demyelination have been used: experimental autoimmune encephalitis (or EAE), and a transgenic mouse with two extra copies of the proteolipid protein (*Plp*) gene (Kagawa et al. 1994). In both models, subsequent to central demyelination, there is a dramatic increase in the amounts of Nav1.2 channels found in the axon (Craner et al. 2003; Rasband et al. 2003). In the case of the *Plp* over-expressor, the amount of Nav1.2 channels in demyelinated optic nerve axons increases by more than 6-fold. The results described here do not appear to be a phenomenon unique to mice. In fact, post-mortem brain tissue from multiple sclerosis patients shows a dramatic increase in Nav1.2 channels in demyelinated lesions (Craner et al. 2004). Taken together, these results all point to the conclusion that myelinating oligodendrocytes directly regulate the kinds of Nav channels that are found in the axon and their localization, rather than the neuron dictating these properties.

3.2

Neuron-Glia Interactions at the Paranode

Since the principle site of neuron-glia interaction is the paranode, it is conceivable that this site may contain the signaling machinery responsible for regulating Nav channel subtype expression and localization. This possibility was tested in the *Caspr*-null mouse. These mice have apparently normal myelin, but fail to form normal paranodal contacts: transverse bands do not form and many paranodal loops are everted and face away from the axon. Importantly, Nav1.2 is still detected at more than 50% of nodes in mature mice, indicating that in the absence of appropriate axoglial junc-

tion formation, Nav1.6 channels fail to completely replace Nav1.2 (Rios et al. 2003).

Several other paranodal mutant mice have been generated through the deletion of enzymes that catalyze the production of the galactolipids galactocerebroside (GalC) and sulfatide. For example, both the [UDP-galactose:ceramide galactosyltransferase-null (CGT-null)] and [cerebroside sulfotransferase-null (CST-null)] have a similar phenotype: aberrant paranodal structure, impaired action potential conduction, and altered ion channel localization (Dupree et al. 1999; Honke et al. 2002; Ishibashi et al. 2002). In many respects, the phenotype of these animals is identical to the Caspr- and Contactin-null mice. While it is easy to understand the structural abnormalities in the Caspr- and Contactin-null mice, it is more difficult to understand how loss of galactolipids leads to the same phenotype. One suggestion to explain these results is that galactolipids may be structural components of paranodes. However, one additional clue is the observation that with the loss of galactolipids, there is a total absence of NF-155 from paranodes (Poliak et al. 1999). To explain these various results, Schafer et al. (2004) showed that NF-155 has important biochemical characteristics of a lipid raft-associated protein. For example, a fraction of NF-155 is resistant to detergent extraction at 4 °C, but becomes soluble in the same detergent at 37 °C. More importantly, this same fraction of detergent insoluble NF-155 floats at very low sucrose densities and becomes soluble when cholesterol is removed from cell membranes. NF-155 acquired these biochemical properties concomitant with paranode formation, suggesting that the recruitment of NF-155 to lipid rafts may depend on binding to its axonal ligand; importantly, both Caspr and Contactin were also recruited to the same lipid rafts and had the same biochemical properties as NF-155. To directly test the possibility that neuron-glia interactions were responsible for the association of NF-155 with lipid rafts, the biochemical properties of NF-155 were examined in paranodal mutants. Although no overall reduction in NF-155 was detected in these animals, NF-155 failed to partition into lipid rafts. Taken together, these results suggest that *trans* interactions between axonal binding partners (e.g. Caspr and/or Contactin) and glial NF-155, and *cis* interactions between NF-155 and myelin galactolipids are important for stabilization and formation of the paranode.

What are the molecular interactions that underlie the paranode? Since Caspr, contactin, and NF-155 are all localized to the paranode, and all have been shown to be important in forming the septate junctions in *Drosophila* epithelia, and since all are cell adhesion molecules, it is not difficult to imagine a tripartite CAM complex underlying the paranode. In fact, in *in vitro* pull-down assays Charles et al. (2002) demonstrated that NF-155-Fc fusion proteins could bind to Caspr and Contactin in lysates from brain. Furthermore, they showed this same NF-155-Fc fusion protein could bind to the surface of cells transfected with Caspr and Contactin, but not Contactin alone, suggesting that all three proteins are involved in neuron-glia interac-

tions at the paranode. In direct contrast to these experiments, Gollan et al. (2003) showed that in vitro NF-155 bound to Contactin alone and that binding of Caspr to Contactin actually inhibited the ability of NF-155 to interact with Contactin. Although the data in Gollan et al. (2003) were quite convincing, one question that remained unanswered is what is the function of Caspr at paranodes if it is not needed for interaction with NF-155? One possibility arises from the fact that Caspr binds to protein 4.1B and that paranodal proteins have properties of raft-associated proteins. If the basis of the paranodal interactions is only through Contactin and NF-155, perhaps this interaction is sufficient to set up the paranodal lipid rafts (both axonal and glial) described above. Contactin-Caspr heterodimers may be recruited into these lipid rafts based on the GPI anchors in Contactin. Caspr would then be appropriately positioned to participate in paranodal cytoskeletal interactions that could further stabilize the paranodal structure. This model relies on neuron-glia interactions establishing an environment into which Caspr-Contactin complexes are recruited. Future experiments will need to determine if single Contactin molecules exist at the paranode or if all Contactin is complexed with Caspr at these sites.

One problem in understanding the specifics of the protein interactions at the paranode is that the detergent insolubility of the paranode makes co-immunoprecipitation experiments difficult. More stringent detergents that disrupt these lipid rafts also appear to disrupt interactions between NF-155 and Contactin/Caspr. It may be that individual paranodal protein interactions are very weak, and that the basis of the strong adhesion complex at the paranode is the sum of these many weak interactions.

3.3

Neuron-Glia Interactions Regulate Juxtaparanodal K⁺ Channel Localization

As described above, a variety of paranodal mutant mice exist. One interesting common phenotype among all of these animals is that Kv1 K⁺ channels, normally restricted to juxtaparanodes (Fig. 2C), invade into paranodal zones whenever transverse bands are absent or paranodal loops are everted (Dupree et al. 1999; Bhat et al. 2001; Boyle et al. 2001; Ishibashi et al. 2002). These observations have led to the conclusion that paranodes also function as a diffusion barrier, to exclude Kv1 channels from paranodal and nodal domains.

While the results above suggest that the neuron-glia interactions at the paranode act as a passive barrier to limit Kv1 channel localization, there is also evidence that myelinating glia actively influence how Kv1 channels are distributed in the axon. One of the first experiments to show this was done using the lyssolecithin model of peripheral demyelination. In these experiments, Rasband et al. (1998) examined the localization of Kv1 channels during remyelination. They found that Kv1 channels are initially not detected at newly forming nodes of Ranvier. However, only a few days later Kv1 chan-

nels can be detected at nodes colocalized with newly formed clusters of Nav channels. These Kv1 channels do not remain in the nodal gap, instead they are redistributed from nodal, through paranodal, and finally into their proper juxtaparanodal locations. When remyelination was inhibited, Kv1 channels accumulated in the axolemma, but failed to be clustered at any particular location.

Consistent with these observations, studies in hypomyelinating and dysmyelinating mutant mice have also shown that Kv1 channel localization is disrupted in the absence of appropriate myelination and junction formation (Wang et al. 1995; Baba et al. 1999; Rasband et al. 1999b). Furthermore, in some mutant mice Kv1 channel expression appears to be dramatically upregulated (Wang et al. 1995). Taken together, these results suggest that myelinating glia actively regulate the locations of both Nav channels and Kv channels.

What molecular mechanisms underlie the neuron-glia interactions that regulate Kv1 channel localization at juxtaparanodes? This question has now been answered through the use of Caspr2 and TAG-1 knockout mice (Poliak et al. 2003; Traka et al. 2003) as well as a more complete picture of the protein interactions occurring at the juxtaparanode. We know that Kv1 channels bind to Caspr2 indirectly, through a yet to be identified PDZ domain containing scaffolding protein (Poliak et al. 1999; Rasband et al. 2002). Caspr2 in turn interacts in *cis* with TAG-1 in the axonal membrane. Surprisingly, the Caspr2/TAG-1 heterodimer binds in *trans* to glial TAG-1 to complete the neuron-glia protein complex. Animals lacking either TAG-1 or Caspr2 have appropriate numbers of Kv1 channels, but these channels fail to cluster at juxtaparanodes. It is important to emphasize that there are no defects in paranodal structure or myelin, pointing to the specific loss of the neuron-glia interaction as the cause of the aberrant localization of Kv1 channels (Poliak et al. 2003; Traka et al. 2003). Taken together, these results demonstrate the essential contribution of neuron-glia interactions in regulating the localization of Kv1 channels to juxtaparanodes.

4

Conclusions

This chapter has focused on several examples that illustrate how neuron-glia interactions regulate the proper formation and function of the node of Ranvier and its associated domains (i.e. paranode and juxtaparanode). Despite the rapid progress that has been made in recent years to define these interactions, much remains unknown. For example, it is interesting to note that virtually every protein component of a node of Ranvier is also found at the axon initial segment. However, in contrast to nodes, axon initial segments do not require any extrinsic input (neuron-glia interactions) to form

high-density clusters of Nav1.6 channels. Thus, intrinsic determinants are sufficient to establish the axon initial segment membrane domains, yet extrinsic factors are required for node of Ranvier formation. What is the interplay between intrinsic and extrinsic factors at nodes? Other intriguing questions may include the following: what is the signaling cascade whereby oligodendrocytes actively regulate the kinds and amounts of Nav and/or Kv1 channels in an axon? How is the positioning of nodes achieved? What are the molecular details of the paranodal axoglial apparatus? And finally, to date we only know of one glial paranodal protein, NF-155. Surely there must be additional proteins found at this site. What are these, what are their functions, and how do they inform the oligodendrocyte or Schwann cell that a paranode has been formed? While all of these questions are very interesting from an academic and scientific perspective and will further our understanding of the nervous system, their answers will no doubt have far reaching consequences as they influence future treatments for the many debilitating and deadly diseases and injuries that result in demyelination and loss of neuron-glia interactions.

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Cognate Interaction Between Endothelial Cells and T Cells

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Abstract Endothelial cells lining the blood vessels form a barrier between circulating immune cells and parenchymal tissue. While the molecular mechanisms involved in antigen-independent recruitment of leukocytes into infected tissue have been extensively studied, the mechanisms involving antigen-specific recruitment of T cells into tissue have remained largely elusive. Here I shall review the experimental evidence that endothelial cells function as antigen-presenting cells and in this function contribute first to regulation of immune responses and second, to antigen-specific recruitment of T cells.

1

Organ-Specific Phenotypes and Functions of Endothelial Cells

The vascular system comprises a network of vessels linking the heart with organs and tissues to maintain homeostasis in response to physiological and pathological conditions. Endothelial cells line the inner surface of blood and lymphatic vessels and show remarkable functional differentiation in diverse microenvironments. They may either form tight, continuous monolayers—such as the blood–brain barrier—or form a discontinuous layer of cells, which allows rapid exchange of fluids and particles to occur. Endothelial cells contribute to the development and modelling of vasculature, blood fluidity, coagulation, nutrient exchange and organ development. Clearly, different organ microenvironments require a different functional range of endothelial cells to support organ-specific functions, to vary blood pressure and to maintain tissue homeostasis. Transcriptome analysis revealed large differences in gene expression in endothelial cells isolated from various sites. Remarkable differences in gene expression patterns were detected for macrovascular vs microvascular endothelial cells, for arterial vs venous endothelial cells and for microvascular endothelial cells from various organs (Chi et al. 2003). These results suggest that endothelial cells undergo extensive functional differentiation in order to support the local physiology of the respective organ. Endothelial cell diversity is further demonstrated by the presence of organ- and tissue-specific endothelial cell receptors, termed vascular signatures, which were identified by phage display analysis (Pasqualini and Ruoslahti 1996).

Differentiation of endothelial cells is driven by the local organ-specific microenvironment and requires continuous “education”, because isolated endothelial cells rapidly lose their functional characteristics and change their gene expression profile (Lacorre et al. 2004). Results obtained from experiments where endothelial cells were serially passaged before functional analysis should therefore be viewed with caution.

The adaptation of endothelial cells to organ-specific functions is enormous and often not only complements a particular organ function, but is also a critical prerequisite for this function. For instance, microvascular endothelial cells of the brain constitute the so-called blood–brain barrier. Endothelial cells forming this interface must ascertain dynamic regulation of ion balance between the peripheral circulation and the central nervous system, facilitate nutrient transport, and function as a barrier to prevent access of potentially harmful molecules or pathogens. Anatomically, endothelial cells of the blood–brain barrier are distinguished from those in the periphery by larger numbers of mitochondria, lack of fenestrations and minimal pinocytotic activity. A number of molecules contribute to tight junction formation, such as JAM-1, claudins, ZO-1 and ZO-2, thus assuring barrier function (Hawkins and Davis 2005). Transport of molecules and access by pathogens to the central nervous system occur via transcytotic transport through brain endothelial cells (Descamps et al. 1996).

Lung microvascular endothelial cells are faced with other functional demands. Normal pulmonary function depends on the maintenance of fluid balance. Given the vast surface area of the pulmonary microcirculation and the vicinity to the alveolar epithelial barrier, where gas exchange occurs, tissue fluid balance must be maintained within the entire organ to prevent fluid leakage into the alveolar space. Lung endothelial cells serve to maintain local vascular permeability, and expression of tight junction proteins is operational in mediating this function (Boitano et al. 2004). However, albumin transcytosis by lung microvascular endothelial cells is important to maintain tissue colloid osmotic pressure and to maintain barrier integrity. The development of pathophysiological changes within the lung is often associated with an activation of endothelial cells, which subsequently triggers shape changes of endothelial cells. These mechanisms may result in increased local paracellular movement of fluid into the interstitial compartment. As a consequence, gas exchange problems are generated which finally may lead to organ failure (Mehta et al. 2004).

Liver sinusoidal endothelial cells represent a family of scavenger endothelial cells, which are most active with respect to scavenging of macromolecules from blood passing through the liver (Smedsrod 2004). Liver endothelial cells show perforations, so-called fenestrae, which allow diffusion of molecules smaller than 20 nm from the bloodstream into the space of Dissé. As the metabolic function of the liver entails both clearance of waste molecules from the bloodstream and extraction of nutrients for metabolic activity, the enor-

mous scavenger activity of liver sinusoidal endothelial cells in part defines the physiological function of the liver. Molecules initially taken up by receptor-mediated endocytosis into scavenger liver sinusoidal endothelial cells are transported subsequently by transcytosis to hepatocytes (Tavassoli et al. 1986). Although the liver endothelium lacks a basement membrane, it appears that these cells constitute a physical barrier that at least restricts access of blood-borne leukocytes to liver tissue (Limmer et al. 1998; Bowen et al. 2004).

Lymphatic endothelial cells are largely responsible for uptake of molecules from interstitial tissue. Although endothelial cells of lymphatic capillaries share many common properties with endothelial cells from blood vessels, they bear a number of distinct structural features that reflect their specific function and allow their molecular characterization (Podgrabsinska et al. 2002). Lymphatic endothelial cells often lack a basement membrane, they contain numerous invaginations and show overlapping intercellular junctions that are only sparsely distributed. Lymphatic endothelial cells are further connected to the extracellular matrix by anchoring filaments, thus integrating these endothelial cells into the interstitium (Scavelli et al. 2004). Apart from participating in interstitial fluid balance regulation, lymphatic vessels serve as an entry and trafficking pathway for antigen-presenting cells on their way from the tissue to lymph nodes.

High endothelial venules (HEV), lining the blood vessels of lymph nodes, function to recruit subsets of T and B lymphocytes from the circulation into lymphatic tissue (Miyasaka and Tanaka 2004). HEV possess a couple of unique features, which are not observed in normal venular endothelial cells. For example, they have a thick basal lamina, a prominent perivascular sheath and a tall and plump appearance. In particular, HEV from peripheral lymph nodes express highly glycosylated and sulphated forms of sialomucins, CD34, podocalyxin, endoglycan and endomucin (Miyasaka and Tanaka 2004). Under physiological conditions, expression of certain chemokines (e.g. CCL19, CCL21, CXCL12 and CXCL13) by HEV is operative in controlling lymphocyte trafficking to lymph nodes and Peyer's patches. It is of interest to note that entry into lymph nodes occurs for antigen-presenting cells mostly via the lymphatic vessels and for lymphocytes through HEV. Both antigen-presenting cells and naïve T cells express identical chemokine receptors, e.g. CCR7, allowing them to meet in certain microenvironments within peripheral lymph nodes.

These examples illustrate the vast functional repertoire of endothelial cells in different organ microenvironments, which is accompanied by characteristic differences in gene expression profiles. As endothelial cells serve so many different functions, it is impossible to draw general conclusions on the immune function of "the endothelial cell". Rather, we should view endothelial cells as distinct organ-specific cells that participate in shaping the local immune milieu required to maintain homeostasis under physiological as well as pathological conditions.

2

Establishment of Interaction Between Endothelial Cells and T Cells

Leukocytes pass through blood vessels at considerable speed, reaching up to 900 $\mu\text{m/s}$. The well-known multi-step paradigm of lymphocyte binding to endothelial cells involves sequential engagement of selectins as well as adhesion molecules and G-protein coupled chemokine receptors, finally resulting in firm leukocyte adhesion to the endothelial cell, which is a prerequisite for subsequent transendothelial migration to enter the tissue (see Fig. 1). A recent report from Shamri and colleagues (2005) clearly illustrated that leukocyte activation and firm binding to endothelial cells required endothelial cell-bound chemokines, occurred within less than a second after initial contact, and involved the induction of a high-affinity extended lymphocyte function-associated antigen-1 (LFA-1) conformation. A detailed analysis of the molecular mechanisms governing leukocyte–endothelial interactions is given in the chapter by Kolanus (this volume).

In general, most cells interacting with endothelial cells are not T lymphocytes but neutrophils. A large body of experimental evidence has been accumulated that describes the different steps involved in initial binding, adhesion, firm tethering and transendothelial migration. Most important to the initiation of this cascade is the activation of endothelial cells through pro-inflammatory stimuli such as tumour necrosis factor alpha (TNF- α) or interferon gamma (IFN- γ), because endothelial cells prevent leukocyte adhesion under steady-state conditions through constitutive release of prostacyclins, NO or adenosine. However, the molecular mechanisms leading to the accumulation of antigen-specific T lymphocytes at local sites of infection and allowing the antigen-specific immune system to eradicate local infection still remain unclear. In the following sections I will review the current knowledge on the cellular and molecular mechanisms that enable endothelial cells to

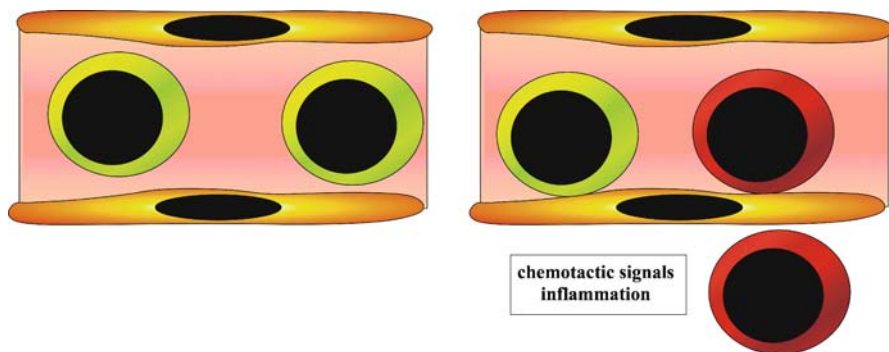


Fig. 1 Mode of T cell – EC interaction. Transmigration of lymphocytes across endothelial cells along a chemotactic gradient

engage in cognate interactions with T cells and the implication of these processes for T cell recruitment.

3

Endothelial Cells as Antigen-Presenting Cells

As endothelial cells form a barrier between leukocytes travelling in the bloodstream and the underlying tissue they are ideally positioned to convey information from tissue to the pool of circulating lymphocytes. Chemokines released from parenchymal cells may be transported by endothelial cells from the basolateral side to the luminal side, and there contribute to antigen-independent recruitment of leukocytes to the vessel wall (Shamri et al. 2005). Endothelial cells further attract leukocytes via expression of certain adhesion molecules, e.g. leukocytes migrate to the skin as a consequence of expression of common leukocyte antigen (CLA) or to the gut and liver via MADCAM-1. To recruit antigen-specific T lymphocytes, endothelial cells need to interact in a major histocompatibility complex (MHC)-restricted cognate fashion with passenger T lymphocytes. In general, the functions of professional antigen-presenting cells, such as dendritic cells, can be divided into at least three different categories: (1) constitutive MHC I and MHC II expression in combination with the ability to efficiently ingest and process antigen; (2) provision of sufficient co-stimulation to prime naïve CD4 or CD8 T lymphocytes; and (3) the induction of T lymphocyte differentiation and function. At least microvascular endothelial cells from some organs appear to fulfil these criteria (Choi et al. 2004; Marelli-Berg and Jarmin 2004; Valujskikh and Heeger 2003), as will be described below.

4

MHC Expression and Antigen Presentation by Endothelial Cells

Microvascular endothelial cells from the human kidney (Muczynski et al. 2003), human heart (McDougall et al. 1997), skin (Vora et al. 1994), gut (Haraldsen et al. 1998) and liver (Lohse et al. 1996) constitutively express MHC class II molecules. In general, most microvascular endothelial cells from humans show constitutive expression of MHC class II molecules, whereas most murine microvascular endothelial cells seem to require pro-inflammatory mediators in order to up-regulate expression of MHC class II molecules. Although this seems to point to a fundamental difference between microvascular endothelial cells from mouse and man, it has to be kept in mind that most animal experiments are normally performed using mice that are kept under specific pathogen-free (SPF) conditions. The observation that even human microvascular endothelial cells rapidly lose their constitutive MHC

class II expression in culture supports the notion that continuous interaction with microorganisms and leukocytes *in vivo* is operational in maintaining expression of MHC-II molecules on endothelial cells, presumably via secretion of IFN- γ from T cells (McDougall et al. 1997). Nevertheless, it is important to keep in mind that most studies using endothelial cells as antigen-presenting cells are conducted with endothelial cells that were pre-stimulated with pro-inflammatory mediators, such as IFN- γ or TNF- α .

Besides expression of MHC molecules, a further prerequisite to function as an antigen-presenting cell is the ability to ingest sufficient amounts of exogenous antigen that is subsequently proteolytically processed in endolysosomal compartments and loaded onto MHC class II molecules. However, little is known about the efficiency of endothelial cells to take up antigens. Whereas dendritic cells employ macropinocytosis in combination with receptor-mediated endocytosis, only certain types of endothelial cells function as scavenger cells and take up significant amounts of soluble antigens from the circulation (Smedsrod 2004). Microvascular endothelial cells from liver and lymph nodes bear numerous scavenger receptors and are most active in eliminating soluble macromolecules from the circulation, thereby accounting for the clearance function of the so-called reticulo-endothelial cell system (Wake et al. 2001). Indeed, intravascular injection of fluorochrome-labelled molecules results in uptake of this model antigen in all sinusoidal endothelial cells in the liver as well as in some microvascular endothelial cells in spleen and lymph node—suggesting that these types of endothelial cells are most efficient in antigen uptake—but not in endothelial cells in the kidney or the lung (P. Knolle, unpublished observation). Given these differences between microvascular endothelial cells from various organs, one would expect that non-scavenger endothelial cells are unlikely to function as antigen-presenting cells. However, since many studies addressing the antigen-presenting capacity of endothelial cells use peptides, alloantigens or superantigens rather than nominal antigens, thus bypassing the need for antigen uptake and antigen processing by endothelial cells, the question as to which type of endothelial cell is indeed capable of antigen presentation remains elusive.

Presumably, the best way to evaluate the antigen-presenting capacity of a certain cell population is to test it for its ability to cross-present exogenous antigens on MHC class I molecules to CD8 T cells. Cross-presentation was originally recognized as a complex and exclusive feature of antigen-presenting cells that is restricted to dendritic cells, macrophages and B cells. Microvascular endothelial cells from pancreas have the capacity to cross-present soluble antigens to CD8 T cells *in vivo* (Greening et al. 2003; Savinov et al. 2003). Moreover, even endothelial cells isolated from murine aorta are able to cross-present exogenous antigens (Bagai et al. 2005). It should be noted, however, that antigen presentation by endothelial cells in these reports always required pre-conditioning via pro-inflammatory mediators or whole-

body irradiation of the animals. This suggests that endothelial cells operate as responder cells and require information from other cell populations of the immune system, such as dendritic cells or macrophages, before they can function as antigen-presenting cells. Consequently, the initial decision on whether or not to mount an immune response is not driven by the endothelial cell itself but rather by professional antigen-presenting cells.

Along the same line, additional reports support the view that endothelial cells fail to function as fully professional antigen-presenting cells (Marelli-Berg et al. 2000a; Kummer et al. 2005). Thus, endothelial cells do not present immunodominant epitopes, which lowers the risk of cytotoxic T lymphocyte (CTL)-mediated immune attack against the endothelium. Although the exact molecular mechanisms underlying the failure to present immunodominant epitopes to CTL remain unclear, it is likely that proteasomal processing and TAP-mediated transportation function in a different fashion in endothelial cells as compared to dendritic cells or macrophages. The detailed analysis of the antigen-presenting capacity of endothelial cells is most relevant, because only cells capable of processing and presenting exogenous antigens are able to communicate information about the presence of infectious pathogens to the immune system. If antigen presentation by endothelial cells was restricted to endogenous antigens, this would be relevant for the development of autoimmunity (towards endogenously presented autoantigens) and endothelitis in organ transplants (towards directly presented alloantigens).

Liver sinusoidal endothelial cells (LSEC) seem to differ from endothelial cells isolated from other organs. LSEC not only function as scavenger cells that efficiently clear the circulation of macromolecules binding to scavenger receptors, mannose receptors and many others, but also bear resemblance to immature dendritic cells with respect to their ability to function as antigen-presenting cells. Exogenous antigens are presented by LSEC in an MHC class II restricted fashion to naïve CD4 T cells. Importantly, in contrast to other endothelial cells, this ability to prime naïve CD4 T cells does not require previous stimulation of LSEC with a pro-inflammatory mediator (Knolle et al. 1999). Although priming of naïve CD4 T cells by antigen-presenting LSEC is less efficient than priming by fully professional antigen-presenting cells such as dendritic cells, it demonstrates that microvascular endothelial cells of the liver are fundamentally different from endothelial cells isolated from other organs (Haraldsen et al. 1998). Similarly, LSEC have the capacity to present soluble exogenous antigens in an MHC class I restricted fashion to CD8 T cells (Limmer et al. 2000). Presentation of antigen by LSEC is a process with considerable efficiency, because even orally ingested antigens are cross-presented by LSEC within two hours after intragastric application (Limmer et al. 2005).

In general, additional studies are needed to evaluate in detail the functional repertoire of the different microvascular endothelial cells in various organs with respect to antigen-presenting cell function. However, isolation of microvascular endothelial cells is experimentally difficult and often leads

to only low numbers of cells. Thus, the discrepant results obtained from different groups working with microvascular endothelial cells often result from important methodological differences in isolation procedures or experimental conditions (Katz et al. 2004). In order to assess the functional repertoire of endothelial cells one must also keep in mind that these cells are organ-resident sessile cells that normally exert their function in a resting, post-mitotic state. If cells are not allowed to adhere they undergo a process termed "anoikis", which is characterized by loss of specific functions and leads finally to apoptosis. During anoikis endothelial cells fail to ingest antigens via receptor-mediated endocytosis and do not display antigen-presenting cell function (P. Knolle, unpublished observation).

Moreover, the unique molecular composition of organ microenvironments may profoundly influence the immune function of organ-resident endothelial cells. This complexity may become even larger in the case of organ damage, inflammation and fibrosis. Only studies that combine well-defined *in vitro* conditions and endothelial cell-specific *in vivo* models will significantly further our knowledge of the immune function of endothelial cells.

5

Co-stimulation by Endothelial Cells

Co-stimulation by antigen-presenting cells is required to stimulate naïve or resting T lymphocytes and may be further categorized in membrane-bound co-stimulatory molecules (such as CD80, CD86 and CD40) or soluble co-stimulation (most importantly interleukin-12 (IL-12)). Endothelial cells from humans (Karmann et al. 1995) and mice (Grewal and Flavell 1998) express CD40 and may thus serve to interact with CD154 (CD40L)-expressing T lymphocytes. The induction of CD154 expression on T lymphocytes is driven through LFA-3 expressed on human endothelial cells and results in a prolonged RNA half-life of CD40L in LFA-3-stimulated T lymphocytes (Karmann et al. 1996). In mice, CD80 and CD86 are the major co-stimulatory molecules (Lenschow et al. 1996); in man LFA-3 and CD80/CD86 seem to be equally important to trigger T lymphocyte stimulation. Human endothelial cells constitutively express LFA-3 (Smith and Thomas 1990) and thus can engage CD2 on human T lymphocytes, but they fail to constitutively express CD80 or CD86 (Denton et al. 1999). In contrast, murine endothelial cells show low-level expression of CD80/CD86, which operates in CD28-mediated co-stimulation of CD4 and CD8 T lymphocytes (Lohse et al. 1996; Marelli-Berg et al. 2000b; Kreisel et al. 2002).

Recently, additional members of the B7 family have been discovered and their expression was detected on human and mouse endothelial cells. B7RP1 (ICOS-L) binds to CD28 on T lymphocytes. The molecule is constitutively expressed on human endothelial cells and functions in co-stimulation of CD4

T lymphocytes, thereby leading to sustained cytokine secretion by CD4 memory T cells (Khayyamian et al. 2002). Another B7 family member is B7-H1 (PDL1), which binds to PD1 on T lymphocytes, a molecule described to function as a negative regulator of T cell activation. B7-H1 is constitutively expressed on murine (Rodig et al. 2003; Freeman et al. 2000) and human endothelial cells (Mazanet and Hughes 2002), and its expression is associated with reduced effector function of T lymphocytes, i.e. reduction of cytokine expression. The expression levels of CD80 and CD86, as well as B7H1 or B7RP1, increase after exposure of human and mouse endothelial cells to pro-inflammatory stimuli. However, the functional consequences of co-signalling molecule expression for T cell stimulation, i.e. co-stimulation or co-inhibition, depends on complex interactions of co-stimulatory molecules on antigen-presenting cells with their respective ligands on T cells. A prediction solely on the basis of expression levels of single molecules is not possible (Chen 2004).

Cytokines released from antigen-presenting cells function as additional co-stimulatory signals and are also most relevant for efficient T cell activation (Curtsinger et al. 2003). Endothelial cells release IL-1 α when appropriately stimulated with pro-inflammatory molecules, such as TNF- α or IFN- γ (Kurt-Jones et al. 1987). In response to contact with viruses, endothelial cells can release type I IFN. Importantly, type I IFN can function as a third signal besides MHC and CD80/CD86 to trigger full activation of T lymphocytes (Curtsinger et al. 2005). More importantly, the expression of IL-12 (Lienen-luke et al. 2000) and IL-18 (Gerdes et al. 2002) has been demonstrated for human endothelial cells. But so far, there are no reports describing the expression of IL-12 by murine or human microvascular endothelial cells, so there is still debate as to whether microvascular endothelial cells can also provide sufficient "signal 3" to drive T lymphocyte differentiation towards immunity.

Taken together, the experimental data support the notion that endothelial cells provide some co-stimulation. But as only the combination of sufficient co-stimulation and the ability to ingest, process and present antigens on MHC molecules renders a cell population capable of initiating antigen-specific immune responses, most endothelial cells have to be seen as non-professional antigen-presenting cells.

6

Consequences of Cognate Interaction Between Antigen-Presenting Endothelial Cells and T Cells

The ability of a cell population to function as an antigen-presenting cell relies on the combination of the characteristics described above: antigen uptake, antigen processing and antigen presentation in combination with qualitative and quantitative aspects of co-stimulation. It is important to note that almost

all the data reported so far on endothelial cells as antigen-presenting cells refer to experimental conditions where endothelial cells were pre-stimulated with IFN- γ or TNF- α , or where the cells were exposed to low-dose irradiation. As low-dose irradiation causes endothelial cell irritation and can even result in endothelial cell damage leading to development of apoptosis (Garcia-Barros et al. 2003), one has to assume that irradiation represents another potent stimulus for endothelial cell activation.

As already described above, only one type of microvascular endothelial cell has been described so far that bears a similarity to dendritic cells and does not require pre-stimulation in order to function as an antigen-presenting cell, i.e. the liver sinusoidal endothelial cell. Organ-resident, sessile LSEC are efficient in antigen uptake, antigen processing and MHC-restricted antigen presentation to CD4 and CD8 T cells. Due to the constitutive low-level expression of co-stimulatory molecules, LSEC stimulate cytokine release and proliferation of naïve CD4 T cells. However, in contrast to dendritic cells, antigen-presenting LSEC fail to promote differentiation of naïve CD4 T cells into Th1 cells. Rather, CD4 T cells primed by antigen-presenting LSEC release substantial amounts of IL-4 and IL-10 (TH2 cytokines) upon antigen-specific re-stimulation (Knolle et al. 1999). We have observed that these T cells exert regulatory function towards stimulation of third-party naïve T cells, i.e. they prevented T cell proliferation induced by antigen-presenting dendritic cells (P. Knolle, unpublished observation). Similar to CD4⁺ T cells, LSEC can prime proliferation and initial cytokine release from naïve CD8⁺ T cells in response to exogenous, soluble antigens. As the presentation of exogenous antigens on MHC class I molecules (termed cross-presentation) was previously believed to be restricted to professional antigen-presenting cells, the ability of LSEC to fulfil this function underlines their intermediate position between non-professional antigen-presenting endothelial and fully professional dendritic cells (Limmer et al. 2000). It is, however, important to note that CD8 T cells that were primed by cross-presenting LSEC developed into tolerant T cells and lost both their ability to release cytokines such as IL-2 and IFN- γ and their cytotoxic activity (Limmer et al. 2000). Thus, despite the fact that LSEC are the only endothelial cell population with significant antigen-presenting capacity, they induce T cell tolerance in CD4 and CD8 T cells.

Induction of CD8 T cell tolerance by LSEC is an active process because it involves initial proliferation and cytokine release before the T cells become unresponsive to further stimulation or tolerant, i.e. fail to release effector cytokines and fail to exert antigen-specific cytotoxicity (Limmer et al. 2000). These experiments revealed that LSEC actively contribute to peripheral immune tolerance. Indeed, the liver has been implicated in the induction of tolerance in a number of experimental systems where antigen was distributed via the bloodstream (Cantor and Dumont 1967). This immunoregulatory function of the liver may be linked to the fact that the liver is the site where the bulk of circulating antigens are eliminated from the bloodstream and

where lymphocytes pass through sinusoidal vessels with a narrow diameter at low blood flow, which forces them into interaction with LSEC. Thus, the anatomic site of the liver seems perfectly suited to modulate the immune response towards circulating antigens.

It is important to note that the induction of CD8 T cell tolerance towards circulating antigens by LSEC is also operative under physiological situations. Indeed, the liver has been suspected to be involved in induction of tolerance towards orally ingested antigens (Callery et al. 1989a). Although the generation of regulatory T cells in gut-associated lymphatic tissue certainly remains the mainstay of oral tolerance, several observations argue that additional tolerance mechanisms have to exist to avoid immune reactivity against innocuous oral antigens. Following ingestion, oral antigens are rapidly taken up from the intestine and are found within 10 min circulating in peripheral blood (Peng et al. 1989). As blood from the gastrointestinal tract first enters the liver via the portal vein, it was assumed that the liver should have a role in inducing oral tolerance. Indeed, the generation of a portacaval shunt prevents induction of oral tolerance (Cantor and Dumont 1967; Callery et al. 1989a).

Within the liver, many different cell populations contribute to induction of tolerance towards orally ingested antigens (Callery et al. 1989b; Watanabe et al. 2002; Watanabe et al. 2003), but LSEC seem to play a particular role as they induce tolerance towards oral antigens specifically in CD8 T cells (Limmer et al. 2005). The tolerance-inducing property of LSEC vs the inflammation-inducing function of professional antigen-presenting cells may be especially important for the development of the CD8-dependent autoimmunity that is sometimes observed after oral feeding of autoantigens (Blanas et al. 1996). Clearly, the rapid systemic distribution of oral antigens certainly leads to uptake of antigens by both LSEC and dendritic cells, which, in the latter case, results in T cell activation in the spleen (Gutgemann et al. 1998). As the site where antigen is first encountered by the immune system determines the outcome of the ensuing immune response, i.e. first antigen contact in the liver leads to tolerance and first encounter in lymphatic tissue leads to immunity (Bowen et al. 2004), it is important to know which populations of antigen-presenting cells first encounter and process antigen and present it to the immune system. Variation in antigen uptake by different antigen-presenting cells or the presence of additional pro-inflammatory stimuli may alter an otherwise tolerogenic situation into an immunogenic situation.

7

Modulation of CD8 T Cell Function by Endothelial Cells

With the exception of LSEC, no data suggest a role for endothelial cells from other organs in initiating antigen-specific immune responses. Rather, the ma-

majority of available data focus on modulation of the function of T cells that had already been primed by professional antigen-presenting cells within lymphatic tissues by endothelial cells. In these settings endothelial cells require a first stimulation by pro-inflammatory mediators or by other non-specific stimulatory events (such as low-dose irradiation) before they can engage in immuno-modulation. The key aspect reviewed in this and the following section is the relevance of cognate interaction between antigen-presenting endothelial cells and previously activated or memory T cells.

By examining the role of endothelial cells during organ transplant rejection it became apparent that endothelial cells are capable of stimulating allogeneic CD8 T cells to differentiate into CTL (Biedermann and Pober 1998). However, compared with other antigen-presenting cells, endothelial cells were less efficient as antigen-presenting cells and required the addition of exogenous IL-2 when co-cultured with T cells. More detailed investigations revealed that endothelial cells only have the capacity to stimulate alloantigen-specific memory CD8 but not naïve CD8 T cells because they cannot provide the appropriate co-stimulatory signals (Dengler and Pober 2000). Similarly, CTL stimulated by human vascular endothelial cells undergo clonal expansion and show considerable endothelial cell-specific cytotoxicity, but often fail to secrete larger amounts of IFN- γ upon antigen-specific re-stimulation (Biedermann and Pober 1998, 1999). The endothelial cell-specific cytotoxicity may be of relevance in pathophysiological situations such as during chronic graft versus host disease. Thus, endothelium-specific CTL from the host can recognize allogeneic endothelial cells resident within the transplanted organ and subsequently attack these cells. This attack could lead to endothelitis (which is a first marker of transplant rejection), loss of blood vessels and may terminally result in organ failure (Biedermann et al. 2002) (see Fig. 2).

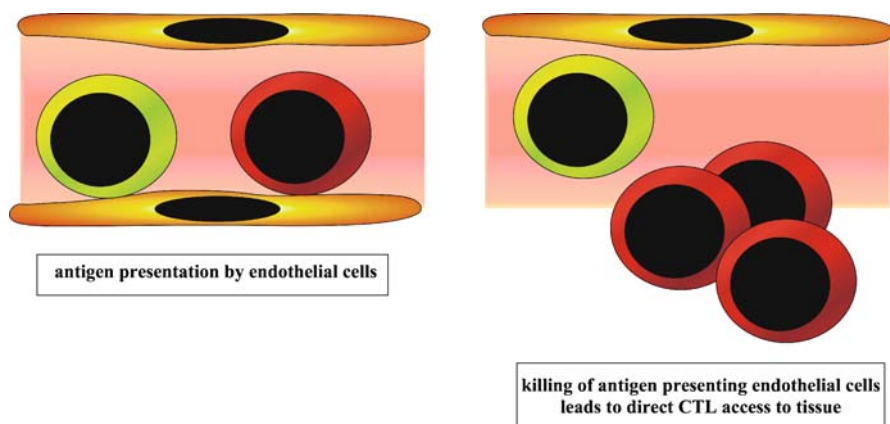


Fig. 2 Mode of T cell – EC interaction. Antigen presentation by EC and recognition by cytotoxic T cells leads to killing of EC and direct access of T cells to the tissue

As antigen presentation predisposes endothelial cells to CTL attack, numerous mechanisms have been described that may be involved in attenuating the target role of endothelial cells. For example, alloantigen-specific CTL stimulated by endothelial cells show strong expression of the activation markers CD69 and CD25 (indicating that they had been activated) but have lower levels of perforin. This may lead to a reduced cytotoxic potential of the activated CTL towards endothelial cells (Dengler and Pober 2000). Furthermore, clonal expansion of CTL induced by endothelial cells is less efficient by a factor of 5–40 compared to T cells that were stimulated by professional antigen-presenting cells which restricts the numbers of CTLs that are capable of attacking endothelial cell specific CTL (Dengler et al. 2001). It is reasonable to propose that both mechanisms operate in concert to limit antigen-specific immune responses against antigen-presenting endothelial cells.

Various additional mechanisms have been described that may further contribute to the immune protection of endothelial cells. Binding of IL-11 to its receptor on human endothelial cells leads to cytoprotection against CTL-mediated attack (Mahboubi et al. 2000). Although the molecular mechanism underlying endothelial cytoprotection is unclear, tyrosine phosphorylation of STAT3 and STAT1 is involved. Moreover, resting murine lung microvascular endothelial cells stimulate proliferation of CD8 T cell clones and to a small extent also naïve CD8 T cells, but cytokine-stimulated endothelial cells fail to promote proliferation of either naïve T cells or T cell clones (Marelli-Berg et al. 2000a). However, in these experiments cytokine stimulation rendered endothelial cells more sensitive towards CTL-mediated killing (Marelli-Berg et al. 2000a). Further evidence indicated that human endothelial cells present a different antigen repertoire than professional antigen-presenting cells (Kummer et al. 2005). In particular, and as already mentioned before, endothelial cells appear to present less immunodominant peptides, which allows them to escape CTL that had been primed by professional dendritic cells (Kummer et al. 2005). Certain microvascular endothelial cells, especially liver sinusoidal endothelial cells, express granzyme inhibitors and thereby escape CTL-mediated killing (Vermijlen et al. 2002). Collectively, all these data support the notion that endothelial cells modulate CTL function in order to attenuate endothelial cell-specific immune responses, and that endothelial cells have developed cell-intrinsic mechanisms to escape CTL-mediated killing.

8

Modulation of CD4 T Cell Function

Similar to the ability to stimulate CD8 T cells, human microvascular gut endothelial cells present complex protein antigens in a MHC class II restricted fashion to CD4 T cell clones (Haraldsen et al. 1998), and the ability of human microvascular endothelial cells to take up and to present nominal antigens

on MHC II molecules was reported in the same study. However, stimulation with IFN- γ was required before endothelial cells could function as antigen-presenting cells. Using the lectin PHA to stimulate T cells, another study showed that human endothelial cells can induce cytokine expression in naïve CD4 T cells (Ma and Pober 1998). However, although this initial stimulation leads to release of IL-2 and IFN- γ , it fails to promote differentiation of naïve CD4 T cells into Th1 cells (Ma and Pober 1998). This suggests that endothelial cells differ from fully professional antigen-presenting cells in their ability to induce TH1 responses, possibly because they do not express co-stimulatory molecules such as CD80/CD86 and also do not release IL-12 (both are hallmarks of classical antigen-presenting cells). Indeed, supplementation of IL-12 to co-cultures enables endothelial cells to fully promote TH1 T helper cell differentiation (Ma and Pober 1998).

Somewhat different results were obtained using allogeneic stimulation instead of lectins. Co-culture of IFN- γ re-activated human endothelial cells with CD4 T cells (mixed lymphocyte reaction) does not initiate a primary allo-response. However, upon allo-specific re-stimulation of T cells an allo-specific non-responsiveness was induced in naïve CD45RA⁺ naïve T cells, whereas reactivity of CD45RO⁺ memory T cells was not affected (Marelli-Berg et al. 1996). The molecular mechanisms responsible for these interesting regulatory capacities of endothelial cells remain unknown. Nevertheless, it is clear that human endothelial cells have the capacity to function as antigen-processing and -presenting cells, but it is also clear that they require pre-stimulation or conditioning before they can exert these functions.

9

Transmigration of T Cells in Response to Antigen Presentation by Endothelial Cells

Lymphocyte re-circulation and recruitment of antigen-specific T cells to sites of inflammation are most relevant for successful immune surveillance (Liu et al. 2004; Engelhardt and Wolburg 2004). The efficiency of T cells in reaching an inflamed tissue is regulated at several levels. During T cell priming in lymphatic tissue by antigen-presenting dendritic cells a number of phenotypic changes, such as surface expression of adhesion molecules, occur that promote T cell migration (Mackay 1993). These pre-activated T cells are then recruited by the endothelium and subsequently are allowed to migrate to sites of inflammation. Receptor-mediated interactions between endothelial cells and T cells regulate this multi-step process. As described above, antigen-independent interactions are operative in recruiting T cells to endothelial cells. The subsequent step of transmigration through the layer of endothelial cells to enter the tissue depends on a number of additional receptor-mediated interactions, e.g. homotypic interactions of CD31 and CD99. Given the abil-

ity of endothelial cells to function as antigen-presenting cells, especially for previously activated T cells, it seems reasonable to assume that additional cognate MHC-mediated interactions between endothelial cells and T cells also promote the recruitment of antigen-specific T cells to sites of inflammation. Indeed, in models of bacterial infection or allergen challenge, an accumulation of pathogen- or allergen-specific T cells was observed at the sites of inflammation (Kawai et al. 1998; Borgonovo et al. 1997). This supports the idea that antigen-specific T cells are indeed preferentially recruited by endothelial cells. Similarly, an accumulation of alloantigen-specific T cells is often observed in organ transplants (Suitters et al. 1990; Zeevi et al. 1986), and Marelli-Berg and co-workers (1999) have shown that MHC class II restricted antigen presentation on human endothelial cells leads to preferential recruitment of antigen-specific CD4 T cells over non-specific CD4 T cells by at least a factor of 4. However, T cell transmigration across an endothelial cell layer that presents the cognate antigen poses a conceptual problem, since it has recently been shown that T cell receptor (TCR) engagement delivers a migration-stop signal to LFA-1-driven T cell migration (Dustin et al. 1997).

This contradiction may be solved by the observation that IFN- γ reverses the TCR-mediated stop signal, thereby allowing migration of antigen-specific T cells into inflamed tissue (Tay et al. 2003). The exact molecular mechanisms that regulate Stop and Go remain unclear, but may entail increased expression of chemokines by IFN- γ -activated endothelial cells or modulation clustering of adhesion molecules at the endothelial membrane (Tay et al. 2003). As endothelial cells in the study by Marelli-Berg et al. (1999) were stimulated with IFN- γ prior to the transmigration experiments, it is likely that IFN- γ was operative in promoting antigen-specific T cell transmigration. It is of interest to note that most endothelial cells require IFN- γ to acquire antigen-presenting cell function, and they also depend on this cytokine to promote transmigration of antigen-specific T cells. The inflammatory milieu as defined by the release of a sufficient amount of IFN- γ is therefore most important to drive recruitment of antigen-specific T cells to local sites of infection (see Fig. 3).

So far, few data are available on the consequences of cognate interaction between endothelial cells and antigen-specific T cells under flow conditions. However, analysis of such events would be most important as T cells rapidly change their shape and migratory behaviour (Shamri et al. 2005) under flow conditions, and may have a different susceptibility for antigen-triggered transmigration compared to steady-state conditions in static adhesion assays. Compelling evidence exists that antigen-presenting endothelial cells preferentially recruit antigen-specific T cells *in vivo*. For example, in the non-obese diabetic (NOD) mouse model adoptive transfer of insulin-specific CD8 T cells leads to development of diabetes (Savinov et al. 2003). In this model, insulin-specific CD8 T cells directly recognized microvascular endothelial cells isolated from the pancreas. Further, expression of CCL21 was also required for efficient antigen-specific transmigration and elicitation

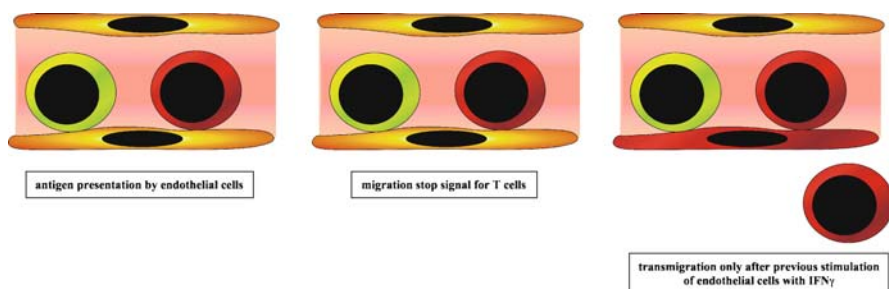


Fig. 3 Mode of T cell – EC interaction. In the presence of soluble mediators antigen presentation by EC may proceed from the migration stop signal for T cells to transmigration across EC

of T cell effector function in the tissue. This study clearly demonstrated that pancreatic endothelial cells have the capacity to cross-present insulin and to recruit insulin-specific CD8 T cells to the pancreas. Although no exogenous IFN- γ was supplied in these experiments, animals were subjected to low-dose irradiation, which is known to lead to a pro-inflammatory environment likely to be rich in mediators such as IFN- γ (J. Alferink, personal communication).

A further report described that circulating HY-specific CD8 T cells are recruited to tissue only in male mice (which carry the HY as an endogenous autoantigen) but not in female mice (which do not express HY). Thus it appears as if cognate recognition of antigen on endothelial cells induces transmigration of antigen-specific cells through the endothelium (Marelli-Berg et al. 2004). Intravital microscopy in the cremasteric vascular bed revealed that cognate recognition of the endothelium results in enhanced diapedesis of T cells into the tissue without affecting rolling or adhesion of T cells (Marelli-Berg et al. 2004). Similar to the reports referred to previously, accumulation of antigen-specific T cells required prior systemic treatment of animals with IFN- γ . Interestingly, preferential transmigration in this model was only observed at some sites, although antigen was ubiquitously expressed in endothelial cells (Marelli-Berg et al. 2004).

There is debate as to whether transmigration of T cells across endothelial cells modulates their subsequent functional repertoire (Nourshargh and Marelli-Berg 2005). It is clear that activated T cells acquire endothelial cell surface determinants during transendothelial migration (Brezinschek et al. 1999) and this may of course influence subsequent T cell function. From the elegant *in vivo* study mentioned above (Savinov et al. 2003) it is, however, also clear that antigen-specific transmigration of T cells does not lead to an alteration in T cell function (with regard to the antigen specificity), because recruitment of insulin-specific CD8 T cells into the pancreas inevitably resulted in insulinitis and development of clinical diabetes (Savinov et al. 2003).

On the other hand, transmigration through endothelial cells seems to augment the functional repertoire of T cells, i.e. augmentation of T cell sensitivity to antigen-specific stimulation and increased motility (Denton et al. 1999; Sancho et al. 1999; Berg et al. 2002). The molecular mechanisms of these functional changes have not been fully elucidated but it is evident that integrin-mediated signalling events are involved, although they are themselves not sufficient to increase the sensitivity of T cells for stimulation (Berg et al. 2002). It is also important to note that the above described functional changes (hyper-motility and hyper-responsiveness) occurred even in the absence of antigen-specific stimulation by endothelial cells. This suggests that trans-endothelial migration of T cells is not strictly dependent on prior antigen-specific stimulation, but amplifies the sensitivity of T cells during subsequent antigen-specific stimulation.

10

Mediation of Antigen-Specific T Cell Recruitment in the Absence of Antigen Presentation by Endothelial Cells

Antigen-specific recruitment of T lymphocytes may not only be achieved through antigen presentation by endothelial cells. Using a model of skin contact sensitivity, it was demonstrated that antigen rapidly enters the blood circulation by diffusion or active transport through tissue and endothelial cells. Minutes after application to the skin, antigen is found to stimulate V α 14J α 18 invariant natural killer T (NKT) cells in the liver to release IL-4, which in turn stimulates peritoneal B1 B cells to express IgM antibodies (Campos et al. 2003). The antigen-presenting cell involved in activation of invariant NKT cells has not been identified, but it is likely that CD1-expressing LSEC and Kupffer cells contribute to this process. Circulating IgM antibodies then form complexes with the antigen at the site of initial antigen application, and trigger local complement activation and release of vasoactive mediators that finally lead to T cell recruitment at the original site of antigen application (Campos et al. 2003). Expression of adhesion molecules, such as CD54 and CD106, by endothelial cells is triggered through local release of vasoactive serotonin and TNF- α , thereby promoting the interaction with effector T cells. This example nicely illustrates the extraordinary position of the liver in scavenging antigens from the systemic circulation and its function as a sentinel organ to detect the presence of foreign antigens. Furthermore, it reveals additional mechanisms that promote antigen-specific recruitment of effector T cells to peripheral sites of inflammation. Circulation of large numbers of effector T cells alone is not sufficient to drive delayed-type hypersensitivity (DTH) responses following antigen challenge; rather, generation of antigen-specific antibodies is required for elicitation of these responses (Askenase et al. 2004).

11

Final Remarks

Cumulative evidence from various *in vitro* and *in vivo* studies reveals that microvascular endothelial cells fulfil different physiological functions in different organs. Microvascular endothelial cells further engage in regulation of the immune response at various levels:

1. They contribute to binding and recruitment of circulating leukocytes in inflammatory conditions through increased expression of adhesion-mediating surface molecules and chemokines. This function occurs independently of antigen-specific interaction.
2. In the presence of inflammation, in particular in the presence of IFN- γ , most microvascular endothelial cells gain antigen-presenting cell function and have the ability to stimulate cytokine release and proliferation of resting T cells as well as memory T cells. However, endothelial cells are in general less efficient antigen-presenting cells as compared to dendritic cells, and thus may contribute to skewing of effector immune responses.
3. Antigen presentation by the endothelium in inflammatory conditions leads to preferential recruitment of effector T cells and may thus improve the efficiency of the adaptive immune response towards pathogens. IFN- γ -mediated signalling is essential for both antigen-presenting cell function and antigen-specific transmigration of T cells, and strengthens the notion that most endothelial cells depend in their immune-regulatory function on a pro-inflammatory environment.
4. In their function as antigen-presenting cells microvascular endothelial cells become themselves the target of antigen-specific immune responses. Obviously, efficient cytoprotective mechanisms must exist to prevent immune-mediated vascular damage.
5. While immune regulation through endothelial cells mostly occurs after T cell responses have been initiated in lymphatic tissue, some microvascular endothelial cells, in particular liver sinusoidal endothelial cells, have the capacity to initiate primary immune responses. However, T cell priming in the liver through antigen-presenting LSEC leads to development of T cell tolerance. As LSEC bear strong phenotypic similarities to lymphatic endothelial cells, more studies are needed to understand the function of lymphatic endothelial cells, especially their function as antigen-presenting cells.

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Impact of the Immunological Synapse on T Cell Signaling

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Abstract T cell activation requires interactions of T cell antigen receptors and peptides presented by major histocompatibility complex molecules in an adhesive junction between the T cell and antigen-presenting cell (APC). Stable junctions with bull's-eye supramolecular activation clusters have been defined as immunological synapses (IS). These structures maintain T cell–APC interaction and allow directed secretion. T cells can also be activated by asymmetric hemisynapses (HS) that allow migration during signal integration. IS and HS dominate in different stages of T cell priming. Optimal effector functions may also depend upon cyclical use of IS and HS.

Keywords Immunological synapses · Signaling · Intravital microscopy · Cell migration · Autoimmunity

Abbreviations

APC	Antigen-presenting cell
CFP	Cyan fluorescent protein
CFSE	Carboxyfluorescein succinimidyl ester
CNS	Central nervous system
DC	Dendritic cell
eGFP	Enhanced green fluorescent protein
HEV	High endothelial venules
HS	Hemisynapse
ICAM	Intercellular adhesion molecule
IS	Immunological synapse
LFA	Lymphocyte function associated
MBP	Myelin basic protein
MHCp	Major histocompatibility complex–peptide complex
NKT	Natural killer T cell
SMAC	Supramolecular activation cluster
TCR	T cell receptor
TIRFM	Total internal reflection fluorescence microscopy
TPLSM	Two-photon laser scanning microscopy
YFP	Yellow fluorescent protein

1

Introduction

The partnership between dendritic cells (DC) and T lymphocytes (T cells) defends the body against microbes, parasites, abnormal cells, and environmental toxins that breach the barrier function of skin and epithelial surfaces (Banchereau and Steinman 1998). Diverse tools including those of biochemistry, cell biology, genetics, and imaging have been employed to understand the mechanistic basis of this partnership. In recent years imaging approaches have become increasingly useful as molecular technologies for labeling cells and proteins, and imaging hardware and software have improved. In vitro imaging led to the initial definition of the immunological synapse (IS) based on cell polarity and segregation of adhesion molecules and T cell antigen receptors (TCR) in the interface between T cells and antigen-presenting cells (APC) (Dustin and Colman 2002). In vivo imaging has led to a basic understanding of the dynamics of T cell-APC interactions in the lymph node and the effect of antigen, which causes formation of long-lived T cell-APC contacts that may be the in vivo counterparts of immunological synapses (Mempel et al. 2004b). Both in vitro and in vivo systems have emphasized the ability of T cells to integrate signals without the formation of a long-lived IS. Therefore, a challenge for in vitro molecular imaging is to provide insight into how T cells integrate signals from IS and more dynamic interactions.

One of the basic biological questions in immunology is what distinguishes T cell responses to DC that lead to tolerance or priming. One concept is that the outcome of antigen presentation depends upon the activation status of the DC (Steinman et al. 2003). Immature DC patrol the tissue spaces and boundaries of the body and gather antigenic structures, both self and foreign. Induced or spontaneous maturation of DC triggers their migration to the lymph node and concurrent processing of antigens to generate peptides that bind to major histocompatibility complex molecules (MHCp), which are then presented at the cell surface. DC migrate to the lymph node via the lymphatics and then migrate in the parenchyma and join DC networks in the T cell zones where they encounter many T cells. The level of costimulatory molecules expressed by the DC is determined by the level of cytokines like TNF produced in response to various endogenous or exogenous activators of innate immunity (Fujii et al. 2004). This level of innate stimulation appears to control whether the antigen-dependent T cell-DC interactions lead to tolerance or priming of an immune response over a period of 5–7 days (Redmond et al. 2003).

Once T cells are primed they can either exit the lymph node to migrate to sites of inflammation or remain within the lymph node to help B cells or contribute to central memory (Lanzavecchia and Sallusto 2002). There has been no work to date on antigen-specific effector T cells in peripheral effector sites, but I will discuss a recent paper on the initial stages of T cell help for B cells

(Okada et al. 2005). There has also been little work on memory T cell interactions with DC during secondary stimulation; however, the availability of mice with stably integrated fluorescent proteins that do not dilute out during cell division should enable such studies.

Peripheral tissue scanning by DC is only one mode of innate immune surveillance of tissues. Two striking examples are the surveillance of the brain by the dynamic processes of microglial cells (Davalos et al. 2005; Nimmerjahn et al. 2005) and the active patrolling of liver sinusoids by natural killer T cells, an innate-like T cell, and leukocytes (Frevort et al. 2005; Geissmann et al. 2005).

In this chapter I will summarize a new view of sustained T cell activation through the IS and the implications of how migrating cells integrate signals through a “hemisynapse” (HS). How the IS and HS work together in T cell tolerance and immune surveillance will then be discussed.

2

New Model for Sustained Signaling Through the Immunological Synapse

Studies on the IS bring together three parallel lines of experimentation in immunology: (1) signal transduction, (2) cell adhesion, and (3) directed secretion.

TCR signaling is based on a tyrosine kinase cascade that leads to rapid activation of phospholipase C γ (Weiss and Littman 1994). The key tyrosine kinases are Lck, which initiates phosphorylation of immunotyrosine activation motifs (ITAM) in the cytoplasmic domain of the TCR, ZAP-70, which is recruited to phosphorylated ITAM, and phosphorylates LAT and ITK, which phosphorylate phospholipase C γ that is recruited to phosphorylated LAT. Phospholipase C γ activation leads to generation of inositol-1,4,5-triphosphate, which induces Ca^{2+} mobilization from intracellular stores and diacylglycerol leading to activation of protein kinase C and Ras exchange factors (Dustin and Chan 2000). The triggering of the cascade is based on recruitment of Lck-associated coreceptors to the TCR and on TCR oligomer formation.

Members of the integrin and immunoglobulin families mediate T cell adhesion to APC. These interactions greatly extend the sensitivity of TCR to small numbers of MHCp bearing agonist peptides (Bachmann et al. 1997). Costimulatory molecules are also configured as adhesion molecules, but have regulatory features that make them less effective as adhesion molecules (Bromley et al. 2001). By definition, adhesion enhances the physical interaction of T cells with APC and the interaction of TCR and MHCp, while costimulation enhances TCR signaling or produces independent signals that integrate with the TCR signal to influence T cell activation. However, the major T cell adhesion molecules have some costimulatory activity. For ex-

ample, LFA-1 contributes to the adhesion of T cells in many contexts, contributes to TCR-MHCp interactions, and provides signals that enhance Ca^{2+} , phosphatidylinositol-3-kinase, and MAPK pathway activation (Dustin et al. 2004). There are also negative costimulators. For example, CTLA-4 and PD-1 negatively regulate T cell expansion at intermediate and late periods of activation (Egen et al. 2002; Okazaki et al. 2002).

Directed secretion is a hallmark of the neural synapses and is one of the most compelling parallels between the IS and neural synapse (Dustin and Colman 2002). Early studies on the mechanism of T cell-mediated killing suggested that killing worked by exocytosis of preformed granules containing lytic molecules with activity-like complement (Young et al. 1986). It was later found that the primary role of perforin was to induce the target cell to take a "poison pill" by introducing granzyme A or B into the cytoplasm, which initiates a proapoptotic caspase cascade (Darmon et al. 1995). Evidence that T cell polarity was related to directed secretion was provided by the seminal studies of Geiger and Kupfer, showing that the microtubule organizing center and Golgi apparatus reorients toward the target cell for killing (Geiger et al. 1982; Kupfer et al. 1983). Kupfer continued with a series of studies on the molecular makeup of the T cell/B cell interface with the first description of CD4, LFA-1, IL-4, talin, and protein kinase C- θ polarization to the interface between T cells and B cells (Kupfer et al. 1986, 1987, 1991; Kupfer and Singer 1989; Monks et al. 1997). All of these studies were performed with fixed cells so temporal information was deduced from populations of images for cells fixed at different times.

In 1998 Kupfer published a paper on the organization of LFA-1, talin, TCR, and protein kinase C- θ in the interface between antigen-specific T cells and antigen-presenting B cells (Monks et al. 1998). LFA-1 and talin were shown to form a ring in the interface, and TCR and protein kinase C- θ were shown to cluster in the middle. These structures were defined as supramolecular activation clusters (SMAC). The TCR cluster marked the cSMAC, while the LFA-1 ring marked the pSMAC. It was implied that TCR signaling was initiated and sustained by the cSMAC. My collaborators and I published a paper in parallel in which live T cells interacting with supported planar bilayers were imaged in real time to visualize segregated adhesive domains composed of LFA-1-ICAM-1 and CD2-CD58 interactions (Dustin et al. 1998). It was posited that the segregation of the adhesion molecules was driven by the different topology of the LFA-1-ICAM-1 (40-nm domain) and CD2-CD58 (15-nm domain) interactions (van der Merwe et al. 2000). The antigen-dependent organization of these domains into a bull's-eye pattern, similar to that reported in several international meetings by Kupfer, was an active process. We proposed the definition of "immunological synapse" (IS) for the bull's-eye pattern described by Kupfer and colleagues and our studies with adhesion molecules, linking a specific molecular pattern to the widely discussed concept (Norcross 1984; Paul and Seder 1994). Taking these two studies together

the IS was defined as a specialized cell–cell junction composed of a cSMAC and a pSMAC (van der Merwe et al. 2000). IS has subsequently been applied to a more diverse array of structures, but we will adhere to this original definition.

The formation of the cSMAC was first evaluated in live T cell-supported planar bilayer models (Grakoui et al. 1999). It was shown that TCR are engaged first in the periphery within 30 s and then these TCR clusters translocate to the center of the IS to form the cSMAC within 5 min. Examination of cell–cell systems showed a similar pattern with peripheral TCR clusters merging in the center to form the cSMAC (Krummel et al. 2000; Lee et al. 2002). This process could take up to 30 min with naive T cells. IS formation is enhanced by CD28–CD80-mediated costimulation (Wülfing et al. 2002), but CD28–CD80 interactions are dependent upon TCR–MHCp interactions (Andres et al. 2004; Bromley et al. 2001; Egen and Allison 2002), perhaps due to induced local rearrangements of actin that are compatible with enhanced CD28–CD80 interaction. Thus, costimulation mediated by CD28 interaction with CD80/86 is a positive feedback loop in IS formation and function.

The IS pattern was highly correlated with full T cell activation *in vitro* in multiple studies using both T cell–B cell, T cell–MHCp, and ICAM-1 bearing planar bilayer and NK cell–target cell IS (Grakoui et al. 1999; Irvine et al. 2002; Monks et al. 1998). While it was recognized early on that the TCR signaling was initiated well before the cSMAC was formed, it was still posited that the cSMAC might be involved in sustained signaling. While kinases can be localized to the cSMAC at 1–5 min, there is a consensus that the cSMAC has relatively low levels of phosphotyrosine, activated phosphoLck, or activated phospho-ZAP-70 at later times (Freiberg et al. 2002; Lee et al. 2002, 2003). Phosphotyrosine staining was retained in the cSMAC at 1 h in CD2AP-deficient T cells. Since CD2AP regulates TCR degradation it was argued that the cSMAC is engaged in continuous signaling, which is made occult by TCR degradation processes (Lee et al. 2003).

A striking property of the cSMAC is that TCR–MHCp interactions in the cSMAC are stable, as measured by fluorescence photobleaching recovery (Grakoui et al. 1999). Since each TCR–agonist MHCp interaction has a half-life of 5–30 s, it would be expected that half of the TCR–MHCp interactions in the interface would exchange free MHCp over a period of several minutes. However, the TCR–MHCp interactions in the cSMAC do not exchange with MHCp in the bilayer over a period of 1 h. The basis of this stabilization is not clear; it may represent a simple avidity effect where MHCp have a very high probability of rebinding the same TCR they have just dissociated from, rather than diffusing away (Lee et al. 2003). I will show later how this dramatic effect can be used to acutely discern differences in signaling between the cSMAC and other structures in the IS. Sustained signaling by T cells appears to be maintained by new TCR–MHCp interactions, since it can be acutely inhibited by antibodies to MHCp that compete for TCR binding (Huppa et al. 2003).

This acute inhibition by anti-MHCp appears to be inconsistent with a central role of stable TCR–MHCp interactions in the cSMAC with TCR–MHCp interactions. However, in the absence of other TCR-containing structures after 5–30 min attention has continued to focus on the cSMAC as a signaling structure, even though the formation of a cSMAC is not required for T cell activation (Lee et al. 2003; Purtic et al. 2005).

To search for other TCR-containing structures in the IS, my lab has employed total internal reflection fluorescence microscopy (TIRFM) with the live T cell-supported planar bilayer system. This is a uniquely advantageous combination for increasing sensitivity to small, low-contrast structures. Through-the-lens TIRFM is based on using very high-resolution oil immersion objectives with a laser focused at the outer edge of the back aperture to steer a shaft of illumination to the sample at an angle that exceeds the “critical angle” (Ax-

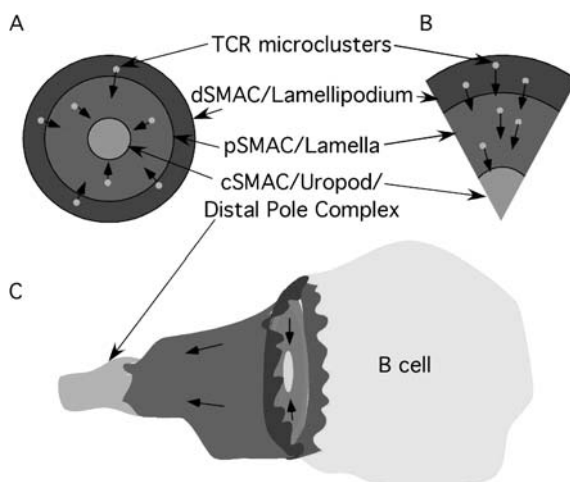


Fig. 1 Modes of T cell interaction deduced from in vitro and in vivo studies. **A** En face view of the IS with cSMAC, pSMAC, and dSMAC as defined by Kupfer (Freiberg et al. 2002; Monks et al. 1998). TCR microclusters as defined by Varma et al. (personal communication) are indicated. Varma et al. propose that these structures mediate sustained signaling. Arrows on schematic indicate direction of TCR microclusters movement. **B** En face view of hemisynapse formed by migrating T cell on antigen-positive APC. In migrating cells the leading edge is a lamellipodium, followed by the lamella and trailed by the uropod. Sims et al. (personal communication) and Varma et al. present data that equate structures of the IS with structures of the HS. **C** Antigen-specific T cells push antigen bearing small resting B cells through the 3D tissue environment (Gunzer et al. 2004). This suggests that IS and HS character can coexist in the same cell with the dSMAC /lamellipodium edge as a line of symmetry. We propose that under these conditions a distinct uropod-like structure will form at the distal pole to the IS. This may be the condition for formation of the distal pole complex discovered by Burkhardt and colleagues (Cullinan et al. 2002). For simplicity the 3D tissue/matrix environment against which the lateral surfaces of the migrating T cells are engaged is not depicted

elrod 1989). Under these conditions all the light is reflected off the interface between the cover glass and the cell, but an evanescent wave is generated that can excite fluorescence within 200 nm of the interface (Fig. 1). This method is difficult to apply at a cell/cell interface because these structures are many microns away from the interface, but it can be very effective for examination of the interface with cells and the supported planar bilayer, which is only 2 nm off the surface of the cover glass (Klopfenstein et al. 2002). Thus, the entire IS can be illuminated with lateral and axial resolution of ~ 200 nm, a uniquely optimal situation in light microscopy. TIRFM is used for single fluorophore imaging so as long as contrast exists, a small fluorescent structure can be detected (Douglass and Vale 2005; Klopfenstein et al. 2002).

Application of TIRFM to the IS led to a striking discovery. While the field had seen the IS formation process as a single wave of TCR-MHCp movement from the periphery to the cSMAC, TIRFM revealed continued formation of TCR-MHCp clusters in the periphery of the IS (Campi et al. 2005; Yokosuka et al. 2005). TCR clustering had been recognized as an important concept on theoretical grounds, based on work with growth factor receptors (Fanger et al. 1986; Schreiber et al. 1983) and the Fc ϵ RI receptor (Erickson et al. 1986; Stauffer and Meyer 1997), but the minimal clusters sufficient to sustain TCR signaling had been assumed to be too small for direct visualization (van der Merwe et al. 2000; Germain 1997). Initially, TCR microclusters can contain up to ~ 150 TCR each (Campi et al. 2005), at which point they are readily detectable by conventional methods, but after the cSMAC forms at 5 min the TCR clusters contain only tens of TCR per cluster and could only be detected by TIRFM (Campi et al. 2005; Yokosuka et al. 2005). Microclusters are detected over the entire range of MHCp densities leading to cSMAC formation (Grakoui et al. 1999; Varma, Campi, Yokosuka, Saito, and Dustin, personal communication). The role of TCR microclusters in signaling was supported by the demonstration that peripheral microclusters were stained with anti-phosphotyrosine antibodies and they recruit ZAP-70-GFP and SLP-76-GFP (Campi et al. 2005; Yokosuka et al. 2005).

Peripheral TCR clusters in the IS appeared to be associated with sustained signaling, but the role of the cSMAC in signaling was still unclear. TCR microclusters only last about 2 min, from their initial formation at the periphery of the IS to the time when they join in the cSMAC, so any treatment that prevents formation of new microclusters would eliminate these structures within 2 min. Varma, Campi, Yokosuka, Saito, and Dustin (personal communication) tested the effect of anti-MHCp antibodies on different structures in the IS. They found that the avidity of TCR-MHCp interactions in microclusters and in the cSMAC is high enough to resist dissociation by antibodies for greater than 10 min, but anti-MHCp Ab blocks new formation of TCR microclusters within seconds. Under these conditions cytoplasmic Ca^{2+} signaling returned to baseline within 2 min. It appears that the persistence of Ca^{2+} elevation for 2 min is related to the time it takes the microclusters that have already formed

to complete movement to the cSMAC. Hundreds of TCR–MHCp interactions in the cSMAC were insufficient to sustain signaling after 2 min. Anti-MHCp also blocked the appearance of ZAP-70 in peripheral microclusters, but did not alter the levels of ZAP-70 associated with the cSMAC (Varma et al., personal communication). These findings collectively redirect our attention from the cSMAC to the more dynamic structures in the periphery of the IS.

Kupfer described the most peripheral part of the IS as a distal SMAC (dSMAC) (Freiberg et al. 2002). The dynamics of the dSMAC are very similar to the lamellipodium of a spreading or migrating fibroblast in that it displays cycles of extension and retraction referred to as contractile oscillations (Giannone et al. 2004; Sims, Xenias, Dubin-Thaler, Hofman, Wiggins, Sheetz, and Dustin, personal communication). The dSMAC is also rich in CD45, the transmembrane tyrosine phosphatase that primes activation of Src family kinases including Lck and Fyn (Freiberg et al. 2002; Johnson et al. 2000). Therefore, actin-dependent TCR clustering by agonist ligands will exclude CD45, setting conditions for rapid tyrosine phosphorylation of partially activated Lck and TCR signaling.

The TCR microclusters traverse the pSMAC, a domain rich in the larger LFA-1–ICAM-1 interaction. The pSMAC is not a solid ring, but a meshwork of micron-scale LFA-1-rich domains with interspersed holes lacking LFA-1–ICAM-1 interaction (Grakoui et al. 1999). The microclusters appear to navigate these holes in a tortuous fashion (Yokosuka et al. 2005). Thus, the TCR nanoclusters do not take a straight-line path to the cSMAC, but zigzag through the pSMAC, perhaps because of the obstacles formed by the dynamic LFA-1 clusters. It is notable that ZAP-70 recruitment and activation appears to be maximal as the TCR microclusters traverse the pSMAC (Campi et al. 2005; Yokosuka et al. 2005). Thus, the close juxtaposition of TCR microclusters and surrounding integrin microclusters appears to be an optimal condition for TCR signaling, but signaling turns off as or shortly after the TCR clusters join the cSMAC.

It has been observed in many studies that when T cells migrate on ICAM-1-containing surfaces they produce “focal zones” of LFA-1–ICAM-1 interaction that accumulate in the lamella, the force-generating structure in amoeboid cell locomotion (Grakoui et al. 1999; Gupton et al. 2005; Heissmeyer et al. 2004; Ponti et al. 2004; Smith et al. 2005; Somersalo et al. 2004; Sumen et al. 2004). These focal zones can vary in shape from crescents to wedges that are analogous to a half or quarter IS. Hypothetically, in a complete IS the inward directed forces generated in the pSMAC are balanced and the cell moves slowly or not at all (Dustin 2004). If half or more of the IS is eliminated then the remaining portion mediates cell movement because the forces generated by the lamella are unbalanced. Thus, I propose that the contact structure used by a migrating T cell to integrate the signal from an APC can be defined as a hemisynapse (HS). HS signal by forming TCR microclusters in the leading lamellipodia, while the microclusters signal as they translo-

cate through the LFA-1-rich focal zone and are inactivated in the uropod, the trailing structure that is analogous to the cSMAC (R. Varma, T.N. Sims, and M.L. Dustin, unpublished observation). This model suggests a way to resolve the controversy regarding IS formation and signaling between groups that typically observe IS formation during T cell activation and those that observe migration only (Gunzer et al. 2000). Since TCR signaling is initiated and sustained in the leading lamellipodium by TCR microclusters, the formation of these structures by migrating T cells is fully compatible with integration of signals. The uropod and cSMAC may differ in that a cSMAC receives and preserves, to some degree, TCR–MHCp complexes and associated molecules, although evidence documented above suggests that TCR accumulated in the cSMAC do not signal, at least not for elevation of Ca^{2+} . In addition, the uropod may only maintain connections using membrane nanotubes, which may prolong signaling connections between immune cells but are likely to break beyond a few tens of micrometers (Onfelt and Davis 2004; Onfelt et al. 2004; Watkins and Salter 2005). The major differences between IS and HS are that IS form a cSMAC, while HS form nanotubes, and T cells forming IS spend more time with the APC than cells forming HS, which move serially from APC to APC.

3

In Vivo Functions of Immunological Synapse and Hemisynapse

TCR signal integration through nanoclusters formed in the IS or HS provides a framework for thinking about the results from recent *in vivo* studies. Since we can now understand the ability of T cells to signal while migrating, we can consider regulation of T cell migration not in terms of signaling changes, but in terms of controlling the network of spatiotemporal cell–cell interactions that recruit different cells into the immune response or allow them to execute effector functions. Another consideration is directed secretion, which is likely best sustained through a stable IS, but may also be executed from a HS for shorter periods.

Naive T cell priming is a central process in initiation of immune responses. Priming of naive T cells requires interactions with dendritic cells *in vivo* (Jung et al. 2002). However, not all antigen-specific T cell–DC interactions will lead to priming. Antigen presentation by dendritic cells in the steady state (absence of inflammation) results in induction of peripheral tolerance (Hawiger et al. 2001, 2004). Presentation under steady-state conditions leads to T cell proliferation followed by induction of antigen-specific nonresponsiveness in the effector cells or their deletion (Vidard et al. 1994). A second mechanism of peripheral tolerance is the induction of antigen-specific regulatory T cells (Mucida et al. 2005). The steady-state process of inducing tolerance preconditions the active peripheral T cell repertoire to react selectively with

pathogen-associated foreign antigens under conditions of infection or tissue damage, since T cells specific for self-antigens and benign foreign antigens are deleted or anergic (Steinman et al. 2003). The dynamics of priming or priming versus tolerance has been the subject of many of the initial wave of intravital microscopy studies.

Stand alone studies on priming of naive T cells have been carried out using three different TCR transgenic models, two different adjuvant systems, and both explanted intact lymph node and intravital imaging approaches. Consistent themes are emerging and hypotheses can be developed regarding the basis for differences between studies.

4

In Vivo Analysis of CD4⁺ T Cell Priming and Tolerance Induction

Stoll et al. (2002) examined the dynamics of T cell priming by transferred mature DC. CFSE-labeled 5C.C7 TCR Tg T cells were introduced into the lymph nodes by intravenous adoptive transfer and DiI- or DiD-labeled DC were introduced into specific draining lymph nodes by subcutaneous injection. 5C.C7 is an I-E^k-restricted TCR that binds moth cytochrome C (MCC) peptide 91–103 as an agonist, so DC were either pulsed with this peptide or a control peptide. The lymph nodes were then explanted, embedded, and imaged with a conventional confocal microscope. This is the only study of lymph node T cell dynamics by conventional confocal microscopy, which limits depth of penetration to tens of micrometers. T cells displayed low basal motility in the absence of antigen, which may be attributed to low perfusion (media movement over the surface of the lymph node). Nonetheless, many more 5C.C7 T cells interacted with antigen-pulsed DC compared to unpulsed DC. The antigen-specific interactions appeared to be stable in that T cells and DC formed extensive interfaces that excluded CD43, a characteristic of *in vitro* IS. Naive 5C.C7 T cells expressing CD43-GFP were prepared by retroviral transduction of bone marrow stem cells followed by reconstitution of irradiate mice. A remarkable transformation took place at 36 h when the activated antigen-specific blasts initiated rapid migration. This 36-h period took place *in vivo*, not in the organ culture, but nonetheless the imaging conditions were the same so it is apparent that recently activated blasts “break” IS-like interactions after 36 h when they initiated rapid migration under conditions where naive T cells were incapable of migration. Subsequent encounters with antigen-positive DC did not lead to IS. This study suggested that CD4 T cells remain in IS until they start to proliferate such that all communication might take place through IS-like structures.

A series of studies by Miller et al. (2002, 2003, 2004a,b) using two-photon laser scanning microscopy (TPLSM) shed light on repertoire scanning and priming in a similar hybrid *in vivo*/explant system, with some modifications

compared to Stoll et al (2002). For information on TPLSM, which allows imaging of cells at depths of up to 500 μm in tissues, I refer the reader to two outstanding reviews (Cahalan et al. 2002; Zipfel et al. 2003). Subsequently in this review, all studies use TPLSM unless otherwise noted. Adoptively transferred DO11.10 TCR Tg T cells were activated with ovalbumin in alum (Miller et al. 2002, 2004b). Alum is an adjuvant that primes Th2 responses, perhaps due to the activation of IL-4 producing APC (Jordan et al. 2004). Unlike the Stoll et al. (2002) system, where the node was immobilized by implanting it in a drop of agarose and imaging it from the immobilized side, Miller et al. (2002) attached the lymph node to the bottom surface of a dish via the hillus and then imaged it from above with a continuous perfusion of highly oxygenated media. These conditions, and perfusion may be the key difference, resulted in the first observations of dramatic motility of naive T cells in the lymph node, which is similar to that observed by intravital microscopy (Miller et al. 2003), and established the current paradigm for repertoire scanning (Miller et al. 2004a). In studies on priming, purified DO11.10 T cells were labeled with CMTMR and adoptively transferred i.v., while DC were labeled in situ by subcutaneous injection of CFSE with the alum/antigen mixture. Therefore, all DC that were present at the injection site and then migrated to the lymph node would be labeled. Resident DC in the lymph node that were exposed to antigen draining via the lymph or immigrant DC that entered the tissue hours after the injection would not be labeled because the reactive dye would be hydrolyzed in a few minutes. It is not clear how effective the alum is at preventing drainage of soluble antigen to the lymph node ahead of immigrant DC, which may have an impact on early priming (Itano et al. 2003). Nonetheless, this was a highly effective method for examining T cell interactions with DC that migrated from the injection site.

The fundamental findings from this series of experiments were: (1) T cells move rapidly and, to a first approximation, randomly in the T cell zones (Miller et al. 2002); (2) similar movement of T cells is observed in vivo using intravital microscopy of the inguinal lymph node (Miller et al. 2003); (3) DC move slowly, but contact many T cells by probing with many veil-like processes, such that each DC can contact up to 5000 T cells per hour (Miller et al. 2004a); and (4) antigen-specific interactions can be more dynamic than those observed by Stoll et al. (2002) with stable interaction between 3 and 16 h, with resumption of antigen-specific swarming and rapid migration by 16–24 h and beyond (Miller et al. 2004b). Even at the most stable phases of interaction the apparent contact area size and the changes over time suggested a more dynamic situation than in vitro IS analysis; however, no molecular imaging was performed. The same model was also used to study oral tolerance in mesenteric and peripheral lymph nodes (Zinselmeyer et al. 2005). In conditions of priming and tolerance T cell clusters were formed and it was assumed, based on the similarity of T cell patterns to those observed by Miller et al. (2004, 2004b), that these clusters formed around DC. Under conditions of priming

the clusters were larger and longer-lived, but stable, IS-like interactions were observed in both priming and tolerance induction. The prevalence of a spectrum of interaction types, from rapid migration to swarming in addition to stable interactions, suggests that HS-like interactions also have a role in *in vivo* priming and tolerance.

Steady-state DC have been visualized *in vivo* using CD11c promoter YFP transgenic (CD11c-YFP) mice (Lindquist et al. 2004). Lymph node DCs include immigrants from the tissues and resident cells that may enter directly from the blood. While tissue inflammation stimulates synchronous migration of many DC to the draining lymph node, there is a poorly understood steady-state migration of DC from the tissues to the lymph nodes that is likely to be critical for peripheral tolerance to tissue antigens. These immigrant DC are mature in that they express high levels of MHC class II on their surfaces, but they are not activated since they only express low to intermediate levels of CD80 and CD86. The CD11c promoter was used to generate a number of transgenic strains that were tested for bright enough fluorescence to be useful in two-photon intravital microscopy at up to 300 μm depth. One founder of several tested had high expression in CD11c^{hi} myeloid DC. In the T cell zones of inguinal lymph nodes of live mice, steady-state DC form extensive sessile networks (Lindquist et al. 2004). Distinct DC behaviors were described in the subcapsular sinus (migrating DC), superficial surface of B cell follicles (layer of dim stationary DC), interfollicular zones (clusters of DC trapping T and B cells), and T cell zones (DC networks). Immigrant DC migrated from the subcapsular sinus to the T cell zones rapidly and join the T cell zone networks. Thus, rapid T cell migration through the DC networks and DC outreach to passing T cells through formation of long membrane processes are the mechanisms that drive repertoire scanning.

CD4⁺ T cell priming and tolerance was studied *in vivo* using the CD11c-YFP Tg mice and a strategy for targeting antigen to DC by attaching antigenic peptides to mAb specific for the scavenger receptor DEC-205 (Hawiger et al. 2001; Shakhar et al. 2005). Under these conditions all of the DEC-205 positive DC, which include CD11c high and low DC types, will present antigen. In the absence of innate immune stimulation, presentation of antigen to the three different MHC class II restricted TCR tested thus far (3A9/HEL, 2D2/MOG, OTII/OVA) leads to tolerance (Hawiger et al. 2001, 2004; Shakhar et al. 2005). Tolerance involves initial activation and expansion of T cells followed by death of the expanded cells between days 3 and 7 after initial activation. Induction of tolerance requires both early exposure to antigen, which induces activation and proliferation, and late exposure to antigen after day 3 to induce deletion (M. Nussenzweig, personal communication).

To perform these experiments, OTII TCR Tg/chicken β -actin promoter-GFP Tg T cells (specific) and non-TCR Tg/CFP Tg T cells (nonspecific) were transferred into CD11c-YFP Tg hosts that had been injected 4 h prior with DEC-205-Ova peptide such that the specific and nonspecific T cells entered

lymph nodes that already contained antigen-presenting DC. Blood flow in the intravital inguinal lymph node preparation was followed using red quantum dots, which excite well at the same TPLSM wavelength. Tolerance was induced with the DEC-205-Ova alone and priming was induced by addition of anti-CD40 mAb to induce activation of DC. This study is distinguished from other studies on CD4⁺ T cells and other studies on tolerance by using intravital microscopy in which blood and lymph flow are intact. Imaging was initiated in three time frames: 0–6, 6–12, and 12–18 h after T cell transfer. Specific T cells showed rapid arrest near high endothelial venules (HEV) within an hour of injection, regardless of whether the conditions favored tolerance or priming. Over the next 18 h the specific T cell regained rapid motility in the DC networks and did not systemically form stable interactions at later times. Some statistically significant differences were detected between tolerance and priming, in that under conditions of priming the rate of return to control migration velocity was slower than that for tolerizing conditions. Since activation and proliferation are induced under both tolerizing and priming conditions, there was a positive correlation between IS-like stable interactions and induction of T cell activation. It appeared that critical signals for tolerance were integrated during later, dynamic HS-like T cell–DC interactions. In fact, continued interaction of T cells with antigen-positive DC appears to be critical for full expansion of CD4⁺ T cells (Obst et al. 2005). Since all three studies on CD4⁺ T cells (Stoll et al., Miller et al., and Shakhar et al.) all concur that T cell–DC interactions are dynamic after proliferation is initiated, it seems very likely that both IS-like stable and HS-like migrating interactions play a key role in CD4⁺ T cells priming and tolerance.

5

In Vivo Analysis of CD8⁺ T Cell Priming and Tolerance Induction

The priming of CD8 T cells has been shown to require a short time of interaction with antigen-presenting cells followed by a long antigen-independent expansion process (Kaeck and Ahmed 2001; van Stipdonk et al. 2003; Wong and Pamer 2001). As mentioned in the previous section, this is very different from the current understanding of antigen requirements for CD4⁺ T cell expansion (Iezzi et al. 1998; Obst et al. 2005). However, induction of antigen-dependent tolerance of CD8⁺ T cells appears to require sustained contact with antigen-positive DC after 72 h (Redmond et al. 2003). Thus, while priming of CD8⁺ T cell responses may be imprinted by early interactions with mature, activated DC, the peripheral deletion of autoreactive CD8⁺ T cells appears not to be imprinted, but to require sustained interactions with DC for longer than 3 days. These biological issues need to be considered in the interpretation of the imaging data.

Bousso and Robey (2003) studied the priming of LCMV gp33 specific P14 TCR Tg T cells using transferred dendritic cells and a similar explanted lymph node imaging approach as Miller et al. (2002). They focused on one time point that was 24 h after transfer. It is assumed that DC migrated rapidly to the lymph node, since they were injected into very proximal subcutaneous sites. Bousso et al. provided the first estimate of DC repertoire scanning rate at 500 T cells per DC per hour. This is tenfold lower than the subsequent estimate by Miller et al. of 5000 T cells per DC per hour, and the major difference may be in the presence of fine dendritic processes that greatly increase the effective target size of the DC in the latter study. This may be an imaging issue or an issue related to handling of the DC altering their morphology. Like Stoll et al., Bousso et al. emphasize the formation of stable IS-like interactions, but only at this one time point. A unique aspect of this study is that it is the only intact lymph node study where antigen dose was varied. They found that stable IS-like interactions were formed over the entire range that would lead to priming.

The first kinetic study of T cell–DC interaction *in vivo* was reported by Mempel et al. (2004a). They performed extensive studies with the P14 line also used before by Bousso and Robey (2003), but they imaged the popliteal lymph node of live anesthetized mice with intact blood and lymph flow. While studies were performed with the DO11.10 CD4⁺ T cells, this data set was very limited compared to the more complete study by Miller et al. (2004b). Mempel et al. used LPS-treated mature CMTMR-labeled DC that were injected into the foot pad, which then drained uniquely to the ipsilateral popliteal lymph node. They injected CFSE-labeled P14 T cells, allowed the cells an hour to enter the lymph node via the HEV, and then injected mAb to L-selectin throughout the rest of the experiment to block further entry. This ensured that the one temporally defined cohort of T cells is followed. The study by Mempel et al. is the first to clearly demonstrate three phases of interaction during priming. Antigen-specific T cells began to encounter DC soon after entering the lymph node, but for the first 8 h the encounters were short lived. These short-lived encounters did activate the T cells since CD69 was upregulated in this time frame. Signal integration in this period (phase 1) suggests HS-like interactions that might involve TCR nanocluster formation. In the 8–12 h time period the contacts were all long-lived IS-like interactions. After this period (phase 2) cytokine production was initiated. At 24–26 h there was a mixed picture of stable, intermediate, and short-lived interaction, and by 44–48 h all interactions were again short lived. This period (phase 3) was characterized by proliferation. Thus, although it was found that CD8⁺ T cells only require a few hours of stimulation to fully commit to many rounds of cell division, the *in vivo* profile of T cell–DC interaction is very similar to what was reported subsequently for CD4⁺ T cells, which integrate signals over longer periods. It is possible that CD8⁺ T cells *in vitro* immediately form IS-like interactions with antigen-rich DC and integrate signals quickly to commit fully to an effector program. In contrast, the sparse antigen-positive DC *in vivo* may

require a longer period of signal integration requiring phase 1 and 2 before becoming antigen independent in phase 3. More work would be required to determine if CD8⁺ T cells do or do not integrate meaningful signals through HS-like interactions in phase 3.

Hugues et al. (2004) studied tolerance versus priming of CD8⁺ OTI TCR Tg T cells using an explant system very similar to that of Miller et al. (2002). They used the DEC-205 antigen delivery approach after CFSE-labeled OTI T cells were transferred, and labeled all DC in the explanted lymph nodes by injecting fluorescently labeled anti-CD11c IgG into the parenchyma prior to imaging. Thus, unlike Shakhar et al. (2005), Hugues et al. delivered antigen to DC after T cells were equilibrated in the lymph nodes, rather than injecting T cells into mice that had been equilibrated with the antigen. They also performed the imaging with explanted lymph nodes in which DC had CD11c and perhaps FcR engaged by anti-CD11c mAb, rather than performing intravital microscopy in a mouse in which DC express YFP. In this study OTI T cells were found to engage in a spectrum of interactions with DC that was biased toward stable interactions in priming conditions and biased to dynamic interactions in conditions of tolerance. Hugues et al. interpreted this set of experiments as indicating that priming involves stable, IS-like interactions, whereas tolerance induction involves HS-like interactions. It is not clear if the differences between the findings of Hugues et al. and Shakhar et al. are due to differences between CD4⁺ and CD8⁺ systems or due to technical differences in the way the experiments were performed. In the study by Shakhar et al. the area around the HEV played a key role in early IS-like interactions, and it might be argued that this region may not function in the same way in the absence of blood flow. Zinselmeyer et al. (2005) used a totally different antigen delivery route and also found that both priming and tolerance involved stable, IS-like T cell migration patterns. It will be important to revisit the issue of T cell–DC dynamics during CD8⁺ T cell tolerance induction in light of these discrepancies.

6

Importance of Stable Interactions In Vivo

Priming and tolerance require T cell activation. Tolerance induction by deletion of effector cells appears to require prolonged TCR signaling beyond 3 days. Thus far, activation is associated with stable, IS-like interaction in nine of ten data sets. Only one data set on tolerance induction is associated with activation, but shows no stable, IS-like interactions, and this is contradicted by two other studies that readily detected stable interactions under conditions of tolerance induction following primary activation. In most cases the formation of stable IS-like interaction is highly correlated to T cell activation, but not to the generation of effector cells or memory, which is only corre-

lated with more subtle changes in the dynamics of interaction. CD4⁺ T cells and CD8⁺ T cells undergoing tolerance induction all integrate signals at a late time (> 24 h) and all show rapid migration with short-duration interactions with DC in this time frame. Thus, migratory HS-like signal integration is likely to be critical for full activation of CD4⁺ T cells and tolerance induction of CD4⁺ and CD8⁺ T cells.

7

Effector Sites

At the time of writing this review, there are only a few papers on intravital microscopy of T cell–APC interactions at effector sites. While there are a number of other studies that report on various lymphoid cells in the intestine and in tumors, I have not included these because there was no concept of antigen-specific interaction; rather, the movement of cell types was documented often with only a limited understanding of the cell type involved. The primary studies that I will discuss have focused on natural killer T (NKT) cells in the liver (Geissmann et al. 2005), helper T cells in the lymph node (Okada et al. 2005), and activated effector cells in the central nervous system (CNS) (Kawakami et al. 2005). These studies all suggest that effector cells form stable, IS-like interactions with antigen-positive APC.

Geissmann et al. (2005) took advantage of the expression pattern of the chemokine receptor CXCR6 in the liver to follow NKT cells *in vivo*. CXCR6 is highly expressed on activated T cells and NKT cells. In the liver of healthy mice NKT cells represent 30% of the mononuclear cells and 70% of the CXCR6⁺ cells. Unutmaz et al. (2000) replaced the major coding exon of CXCR6 with GFP by homologous recombination. The CXCR6^{gfp/+} mice have a normal number of NKT cells in the liver, which are GFP^h, whereas the CXCR6^{gfp/gfp} mice have three- to fivefold reduced numbers of NKT cells in the liver, which remain GFP^{hi} (Geissmann et al. 2005). The reason for the reduced numbers of NKT cells in the CXCR6^{gfp/gfp} mice appears to be reduced NKT cell survival in the absence of CXCR6. The high levels of GFP expressed in the NKT cells afforded the opportunity to track these cells in the liver of mice by intravital microscopy and to determine the effect of antigen. It had been reported that a population of leukocytes identified as Kupffer cells migrated within the sinusoids of the liver (MacPhee et al. 1992). Geissmann et al. discovered that Kupffer cells stained by high molecular weight rhodamine dextran are stationary cells, while NKT cells migrate rapidly within the sinusoids with or against blood flow. This rapid migration within the fenestrated sinusoids allowed wild-type NKT cells to visit each hepatocyte in the liver on average every 15 min. Geissmann et al. never observed GFP⁺ cells extravasating. NKT cells have a dominant V α 14 rearrangement that produces a high-affinity TCR for CD1d with α -galactosylceramide (AGC), which corres-

ponds structurally to a class of bacterial lipids (Kinjo et al. 2005; Mattner et al. 2005). When AGC was injected i.v. most of the NKT cells became activated within 2 h based on cytokine production, and 60% of the GFP⁺ cells stopped migrating and formed long-lived IS-like interactions within 20 min (Geissmann et al. 2005). NKT cells have a previously activated memory/effector phenotype *in vivo*, such that the rapid IS-like response provides the first evidence of interactions with IS-like stability in the effector phase of an immune response. We have also found that conventional effector T cells patrol liver sinusoids (T. Cameron, unpublished observation). This suggests that patrolling of sinusoids is typical of immune surveillance by activated T cells in the liver.

Okada et al. (2005) studied the T cell–B cell interactions during recognition of hen egg white lysozyme (HEL) or I-A^b-HEL 74–88 complexes by MD4 IgG Tg B cells and TCR7 TCR Tg T cells, respectively. The mice were immunized with HEL in alum and explanted lymph nodes were imaged exactly as in the report by Miller et al. (2004). T cell proliferation was extensive by day 2, whereas B cells did not proliferate extensively until day 3. The B cells slowed down in the first 1–3 h and then regained speed and migrated toward the junction between the T zone/B zone interface. This migration was guided by CCL21 gradients in the follicle and required CCR7 expression on the B cells. This is the first demonstration of chemokine-directed migration in an intact lymph node. By 30 h the B and T cells are activated, and long-lived antigen-specific T–B conjugates begin to form at the T zone/B zone interface. The interactions are strikingly different than priming T cell–DC interactions in that the B cells continue to migrate rapidly and drag the T cells behind them. This could be explained by the T cell forming an IS-like stable interaction with the B cell via the B cell uropod, but the B cell continuing to migrate under the influence of chemokinetic and chemotactic factors in the environment. This is also different from an earlier study in which activated T cells interacted with naive B cells, as viewed by conventional confocal microscopy in the inguinal lymph node (Gunzer et al. 2004). In this case the activated T cells pushed the B cells, which rounded up and became sessile after T cell contact. While the study of Gunzer et al. (2004) opens an interesting physical possibility that the lateral surfaces of a T cell can function in migration while the front part forms an IS, it is less physiological than the findings of Okada et al., who studied a classical T cell-dependent antibody response. Again, effector cells form stable IS-like interactions rapidly after encountering APC.

Kawakami et al. (2005) studied the dynamics of T cells in acute spinal cord sliced from rats with early experimental allergic encephalitis (EAE) lesions. The EAE lesions were induced by injecting five million GFP⁺ myelin basic protein (MBP)-specific cloned T cells i.v. Acute spinal cord slices were prepared 4 days after T cell transfer, at which time antigen-specific and non-antigen-specific ovalbumin-specific cloned T cells were also found in the

lesions. This is characteristic of inflamed sites, which are equally attractive for extravasation of antigen-specific and non-antigen-specific cells. Tracking of MBP-specific T cells revealed that 40% were immobile over long periods whereas only 5% of ovalbumin-specific cells were similarly stationary. The remaining cells were migrating in the living brain tissue. Staining of the live sections in real time with antibodies to LFA-1, TCR, and MHC class II revealed polarization of these molecules toward the shared interface between MBP-specific T cells and class II positive APC, but rarely with Ova-specific T cells. While these images were not of sufficient resolution, nor were the staining methods really refined enough, to detect SMACs, nonetheless, evidence for IS-like structures in the tissue sections was compelling. Prior histological analyses in lymph nodes (Reichert et al. 2001) and in the meninges during the CD8⁺ T cell response to lymphocytic choriomeningitis virus (McGavern et al. 2002) have also detected IS-like structures in fixed tissue, but the study by Kawakami et al. (2005) was the first to be able to reference this staining to the dynamics of interaction in *ex vivo* live tissue. While the use of intact antibodies could be criticized, for example, Fab fragments with nonblocking Ab would be a better choice, this study provided a strong step toward imaging of SMACs *in vivo*.

Innate immune surveillance of the CNS was studied by Davalos et al. (2005) and Nimmerjahn et al. (2005). microglial cells are the innate immune cells of the central nervous system in the steady state. These cells can be visualized in live mice in which the CX3CR1 chemokine receptor's major coding exon is replaced by eGFP. Microglial cells are the only cells in the CNS that express GFP in CX3CR1^{+/gfp} mice. Both of these studies used the thinned skull technology developed by Gan and colleagues (Grutzendler et al. 2002) to image microglial cells in the intact brain of anesthetized mice. Parenchymal microglial cells are referred to as "ramified" because each cell projects many long processes in three dimensions to cover a territory of 65 000 μm^3 . The processes extend and retract at a velocity of 1–2 $\mu\text{m}/\text{min}$. Focal injury in the CNS results in a rapid response by the ramified microglial cells in which the cell bodies remain in place, but all the processes within a radius of 75 μm from the site of injury, i.e., dozens of individual processes, converge on the site while maintaining tethers to the cell bodies. While the biological function of this response is not known with certainty, the released ATP is necessary and sufficient to trigger the response, and a reasonable biological hypothesis is that the cells are sealing off local injuries within 30 min. This does not appear to be a classical phagocytic response, and in fact classical phagocytes like neutrophils and monocytes appear to be excluded from small injuries that are completely surrounded by microglial foot processes (J. Kim, unpublished observations). Whether microglial cells that respond to focal injuries are competent to present antigen to CD4⁺ or CD8⁺ T cells is not known.

8

Summary

Recent evidence suggests that the basic units of sustained T cell receptor signaling are small TCR clusters or microclusters dynamically generated near the leading edge of migrating T cells forming HS-like structures, or in the dSMAC of T cells forming stable IS-like structures. Retention of many TCR in the cSMAC is only possible when the T cell stays with one APC, which requires a stop signal, but even then the peripheral TCR microclusters are required to sustain signaling. The specific function of the cSMAC is unknown, but it is not sufficient to sustain Ca^{2+} signals in T cells. In vivo, activation of T cells associated with activation and tolerance requires both stable IS-like and migratory HS-like interactions. IS-like interactions are a common feature of the effector phase and these interactions are initiated rapidly after antigen recognition.

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The Biophysics of T Lymphocyte Activation In Vitro and In Vivo

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Abstract T cell activation is crucial for the development of specific immune reactions. It requires physical contact between T cells and antigen-presenting cells (APC). Since these cells are initially located at distinct positions in the body, they have to migrate and find each other within secondary lymphoid organs. After encountering each other both cells have to maintain a close membrane contact sufficiently long to ensure successful signaling. Thus, there is the necessity to temporarily synchronize the motile behavior of these cells. Initially, it had been proposed that during antigen recognition, T cells receive a stop signal and maintain a stable contact with APC for several hours when an appropriate APC has been encountered. However, direct cell observation via time-lapse microscopy in vitro and in vivo has revealed a different picture. While long contacts can be observed, many interactions appear to be very short and sequential despite efficient signaling. Thus, two concepts addressing the biophysics of T cell activation have emerged. The single encounter model proposes that after a period of dynamic searching, a T cell stops to interact with one appropriately presenting APC until signaling is completed. The serial encounter model suggests that T cells are able to collect a series of short signals by different APC until a critical activation threshold is achieved. Future research needs to clarify the relative importance of short and dynamic versus long-lived T cell–APC encounters for the outcome of T cell activation. Furthermore, a thorough understanding of the molecular events underlying the observed complex motility patterns will make these phenomena amenable for intervention, which might result in the identification of new types of immune modulating drugs.

Keywords Biophysics · Cell–cell interaction · DC · T cell activation

1 Introduction

T lymphocytes are positioned at the crossroads of the human immune system. They are central for efficiently mounting an adaptive immune response. To fulfill their tasks, T cells undergo a complex maturation process and display a characteristic migratory pattern where they physically and functionally interact with many cell types of the body. Thus, for proper functioning of

the immune system, the ability to migrate in an autonomous fashion is an indispensable prerequisite (Friedl and Brocker 2002). In the case of the *de novo* induction of a specific immune response, two diverse cell types, a T cell and an antigen-presenting cell (APC; mainly dendritic cells (DC), B cells, or macrophages (M ϕ)), have to physically engage and need to exchange specific signals. T cells recognize a complex of a peptide antigen in combination with a major histocompatibility complex (MHC) molecule on the surface of APC via their antigen-specific T cell receptor (TCR). During thymic development, the clonotypic TCR are generated by a chance recombination of a huge number of genetic building blocks, also including mechanisms to add variety. This is done by each developing T cell individually, until a functioning TCR is obtained. Thus, although each T cell expresses only one type of TCR, the diversity of the TCR in the whole T cell compartment is immense (Nikolich-Zugich et al. 2004; Goldrath and Bevan 1999). However, although there are millions of different TCR, each one is able to recognize a spectrum of peptide-MHC complexes with different affinity; thus, each antigen will be recognized by a number of individual T cells, albeit with distinct efficiency. This system may help in finding a balance between the ability to recognize a broad set of antigens and to mount a specific immune response in a short enough time (Nikolich-Zugich et al. 2004).

Only contacts with peptide-MHC complexes of sufficiently high TCR affinity will ultimately lead to an activation-associated change in one or both partners, e.g., blast formation and proliferation in T and B cells, or antibody production and antibody class switch in B cells only. However, all cell types that need to interact initially reside in very diverse areas of the body. Moreover, only very few of a body's APC will carry the specific antigen looked for by a specific T cell. In contrast to hormonal signaling, which allows communication between far apart cells by means of small, transportable molecules, from all that we know it is not possible to communicate between an APC and a T cell to indicate which antigen has entered the body, other than by direct physical cell-cell contact. We will see later how, despite these obstacles, the body ensures efficient T cell activation leading to a successful immune response by creating unique immunological "marketplaces" of intense cell communication and cell-cell contact: the lymph nodes. To better understand the functional importance of the migration and cell-cell interactions of T lymphocytes, we will first follow a developing T cell along its way from the bone marrow to the thymus, where precursor T cells prepare for their central task, the encounter with a foreign antigen and the appropriate response to it. The activation of T cells by antigen occurs in the lymph nodes, the anatomy of which we will therefore briefly discuss. Mapping of the T cell's workplace will then open the way for having a closer look at experimental data obtained from direct observations of cell-cell interactions, and at the mechanistic models generated from these data that try to explain how cellular contacts will enable a T cell to become activated *in vivo*.

2

The Role of T Cells Within the Immune System: A T Cell Biography

2.1

T Cell Selection in the Thymus

Like all hematopoietic cells, T lymphocytes originate in the bone marrow. As T cell precursors, they leave the bone marrow via the bloodstream to begin a rigorous two-step test for functionality and self-destructive potential in the thymus, termed positive and negative selection. Details of the thymic selection processes have been described in a number of excellent reviews (Goldrath and Bevan 1999; Sebzda et al. 1999; Correia-Neves et al. 2001). Very briefly, thymocytes must try to establish contact via their then only half-completed TCR with MHC molecules on specialized cortical thymic epithelial cells and DC, respectively, which both present a large variety of self peptides. During these interactions the T cell precursors are sorted by the strength of signals resulting from binding of their TCR to the peptide-MHC complexes presented in the thymus (Bousso et al. 2002; Richie et al. 2002). T cells are positively selected as a result of a weak TCR signal, whereas no signal results in death by neglect and a too strong signal results in negative selection (Alberola-Ila et al. 1995).

For effective interactions with stromal cells, thymocytes decrease their motility, and establish sustained calcium oscillations that may enhance the expression of genes enabling positive selection (Bhakta et al. 2005). The signaling strength mediated by the TCR is believed to be the key to both positive and negative selection. Thus, T cells are selected for survival if the signals received from antigen-presenting DC residing in the medulla of the thymus do not exceed a certain threshold. If they do, suspicion is raised that this high affinity toward the selecting peptide might pose a risk for this T cell to later develop into an autoreactive T cell, thereby priming the organism for the development of autoimmune disease. Consequently, the potentially dangerous T cell is driven into apoptosis. The duration and strength of the signal also participate in the decision as to which of the two main T cell lineages a particular T cell will belong in the future: to the CD8⁺ (killer) T cells bearing direct cytotoxic potential or to the CD4⁺ (helper) T cells providing support to CD8 cells for killing, to B cells for producing antibodies, and to macrophages/monocytes to kill intracellular pathogens such as listeria (Yasutomo K et al. 2000). While there is evidence that positive and negative selection occur spatially distinct from each other and consecutively in time (Bousso et al. 2002; Witt et al. 2005), both processes are aimed at achieving a single goal, namely to provide the highest possible number of T cell specificities available for fighting invaders while minimizing the risk of attacking self tissue. This process is rigorous: more than 90% of all T lymphocytes entering the thymus fall prey to it and die without ever leaving the thymus (Palmer 2003).

2.2

T Cell Circulation and Homeostasis

At the end of the thymic selection process, the now mature naïve (i.e., antigen-inexperienced) T cells are released into the blood and circulate through the periphery (Butcher and Picker 1996). This process comprises the migration of T cells through secondary lymphoid organs and peripheral nonlymphoid tissues. The persistent recirculation is fundamental to the maintenance of immune surveillance. A variety of proteins on the T cell surface facilitate their interaction with resident endothelial, epithelial, stromal, and immune cells and with matrix components of secondary lymphatic organs such as spleen, lymph nodes, and the lymphoid tissue of the respiratory tract and intestine (Butcher and Picker 1996; Potsch et al. 1999; Moser and Loetscher 2001). Initially, naïve T cells will randomly enter secondary lymphoid organs (e.g., lymph nodes) (Mackay 1993; Bradley and Watson 1996)). Upon scanning the environment for a cognate antigen, T cells will predominantly encounter self peptides with which they undergo serial antigen-unspecific, short-lived contacts providing subthreshold survival signals and being implicated in T cell homeostasis (Brocker 1997). It could be shown that recirculating naïve T cells constitutively contact high numbers of DC as they migrate through the perifollicular regions of the lymph node (Miller et al. 2004a). The lymph node is also the place where the most important cell–cell interactions occur with respect to proinflammatory T cell activation toward antigens of invading organisms. Here, antigen drained from the site of infection/inflammation arrives within afferent lymphatic vessels, in soluble form or bound to APC, mostly DC (Itano et al. 2003). The close proximity in the lymph node of foreign antigens, APC, and T cells is essential for the transformation of a resting naïve T cell into an activated, armed effector T lymphocyte.

2.3

T Cell Activation in the Lymph Node: A Marketplace Analogy

In order to get activated, T cells need to find APC with the correct peptide–MHC complexes on their surface. However, there exists an intrinsic information problem. A T cell does not have any knowledge of whether the antigen for which its TCR is specific has been taken up somewhere in the body. Nor does a DC know which T cell is specific for the pathogen that it just had phagocytosed, and whose processed peptide antigens are now presented on its MHC molecules. Likewise, a B cell cannot tell which T cell can provide help for the antigen that fits into its B cell receptor (BCR), a membrane-bound version of the antibody that is being produced by this particular B cell.

The way nature has solved this problem is probably best explained by analogy with a town's marketplace. A marketplace is a defined region where all the town's inhabitants go to check what is being offered by the merchants. Mer-

chants go to this place and set up their booths, because they know that they will have a very high chance of making contact with the town's inhabitants. Inhabitants entering have a preference for specific goods, but they don't know who is selling this particular product. Therefore, they have to migrate over the entire marketplace and interact with many merchants to check the goods. As a result, two individuals who did not know of each other's existence before, and were initially far apart, meet and interact fruitfully.

Transferred to the immune system, the marketplace is a lymph node, the merchants would be APC, and the customers would be T cells. The "goods" that are being presented are self and foreign peptide antigens, which are loaded onto MHC molecules of the APC. Therefore, powerful APC such as DC, which carry $1-8 \times 10^6$ MHC complexes on their surface (Cella et al. 1997), most likely present several hundred if not thousands of different peptides, and thus can serve more than one distinct T cell clone (Fig. 1) (Cella et al. 1997; Kedl et al. 2000).

However, it is not only the T cell that moves toward the lymph node; DC may travel a long way, too. DC usually reside in peripheral tissues of the body and form a tight network of gatekeepers for invading foreign antigens (Fig. 2) (Mellman and Steinman 2001). They leave their position once they have taken up a sample of this antigen by phagocytosis and start a migration process, which ends when the DC reaches the lymph node draining the area of infection, usually 1–2 days later (Itano et al. 2003). This process has been particularly well documented for Langerhans cells (LC), the DC of the epidermis (Romani et al. 2001). Activating tissue-resident LC, e.g., by painting contact sensitizers on the skin or simply by explanting skin and placing it in culture, induces the rapid emigration of skin-resident LC into the lymph vessels of the dermis (Lukas et al. 1996) and from there into draining lymph nodes (Mehling et al. 2001).

The lymph node architecture comprises an inner medullary area, rich in plasma cells and macrophages, and an outer cortical region. The node is surrounded by a sinus and then a capsule. Afferent lymphatics drain into this sinus, thereby bringing new antigens to the node for presentation to T cells (Itano et al. 2003; Gretz et al. 1997, 2000; Ushiki et al. 1995). An unstimulated node's cortex contains primary follicles which are mainly composed of B cells and some DC surrounded by a DC-rich T cell zone. Lymphocytes entering the lymph node from the bloodstream exit the blood vessels by crossing the walls of specialized postcapillary or high endothelial venules (HEV), around which DC may line up in strategic positions to immediately interact with newly arriving T cells (Mempel et al. 2004; Bajenoff et al. 2003). In fact, DC will be the major antigen presenters as they are also the most effective professional APC (Fuchs and Matzinger 1992). However, B cells may also actively participate in antigen presentation if they were able to internalize soluble antigen via their B cell receptor, process the antigen and load the generated peptides onto MHC molecules, and then transport these MHC-peptide complexes to the cell surface (Rodriguez-Pinto and Moreno 2005). B cells will then migrate with di-

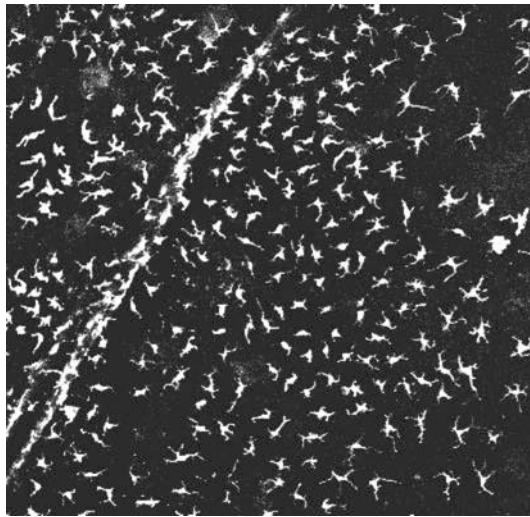


Fig. 2 Langerhans cells, the dendritic cells of the skin, form a tight meshwork of cells in the epidermis of a mouse ear

Immigrant T cells will start scanning the surrounding APC for a peptide-MHC complex capable of binding with high affinity to their TCR. By doing this, they display an interesting and diverse spectrum of biophysical interactions with each of these types of APC (Mempel et al. 2004; Gunzer et al. 2004), which will be looked at in detail below. If the search is finally successful, the T cell will respond to the new situation with proliferation and differentiation into a primed effector cell. For $CD4^+$ T cells, this would be a T helper cell whose main function is to support B cells, whose presented peptides are recognized, to produce antibodies corresponding to the protein from which these peptides were derived. Another important function of helper cells is to support the generation of cytotoxic effector cells. Consequently, a part of the preactivated $CD4^+$ T cell will march toward the lymph node's B cell zone (Okada et al. 2005), while others stay in the T cell zone or leave the lymph node to function in the periphery (Hwang et al. 2004). A $CD8^+$ cell, in contrast, would turn into a cytotoxic T cell (CTL) possessing various direct and indirect possibilities of killing infected cells. In the much more frequent case that a T cell will fail to meet an appropriate antigenic partner, the T cell will leave the lymph node and restart circulating in the body (Butcher and Picker 1996).

2.4

Homing of Activated T Cells

Following a successful activation, T cells differentiate into effector cells with a certain tissue-specific phenotype guiding their homing preferences

(Butcher and Picker 1996; Kannagi 2002; Springer 1990, 1994). Key factors that collectively determine the homing of leukocytes are interactions between integrins or selectins and their tissue adhesion molecules, as well as interactions between chemokine receptors and their specific ligands, the chemokines (Butcher and Picker 1996; Kannagi 2002; Springer 1990, 1994). In addition, the presence of inflammatory stimuli modulates the homing behavior of immune cells (Hwang et al. 2004). Activated T cells start homing to peripheral tissues, where they exert proinflammatory regulatory effector functions or recirculate as memory cells (Sallusto et al. 2004). At the target location, T cells leave the vessels and enter the local tissue ready to exert their effector function (Butcher and Picker 1996; Gunzer et al. 2005). Alternatively, antigen-primed T cells might become memory cells, an option which may occur early during T cell development as well as after differentiation into an effector cell (Seder and Ahmed 2003). Memory cells either reside centrally in lymphoid tissues or in the periphery, with a very high likelihood of expanding rapidly upon reencountering the stimulating antigen (Sallusto et al. 1999).

2.5

T Cell Regulation

A special case emerges when the T cell/APC contact does not generate a full T cell activation status aimed at performing the optimal immune response, but rather results in T cells suppressing the immune response. It is currently a matter of debate as to whether a regulatory cell is a differentiation status of any given T cell, or rather a specific type of T cell that is preferentially expanded in situations where suboptimal activation is prevailing. Evidence for naturally occurring regulatory cells has been obtained (Jonuleit et al. 2001). However, antigen presentation by immature and semimature DC *in vivo* might lead to incomplete T cell activation and *de novo* induction of regulation (Jonuleit et al. 2001; Probst et al. 2003). Regulatory T cells have attracted great interest in recent years and certainly play a very important role in maintaining an efficient and safe immune system (O'Neill et al. 2004; Sakaguchi 2005). The role of cell–cell interactions during this process has not yet been studied in detail (Fontenot et al. 2005).

3

The Biophysics of T Cell Activation

The direct microscopic observation of T cells revealed that upon migration, T cells (which appear round in liquid cell culture) show a very dynamic cell shape with clear polarity: a tail is formed by a motile uropod, as well as a head consisting of the cytoskeletal leading edge by which cell–cell contacts are ini-

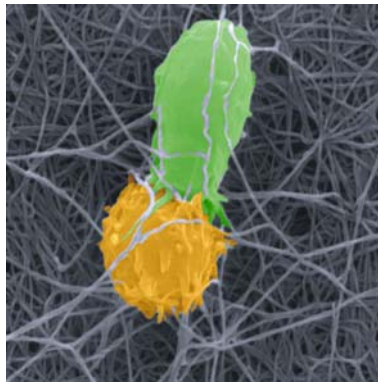


Fig. 3 A naïve T cell (*green*) contacting an antigen-specific naïve B cell (*orange*). Note the intense cell–cell contact as well as the membrane protrusions of the T cell engaging the B cell. The fibrous structures are protein strands consisting of collagen type 1, which was used to simulate a 3D environment. A dynamic aspect of such cell pairs can be obtained from the supplemental material of Gunzer M et al. (2004) on the web page of the journal *Blood*

tiated (Fig. 3) (Mempel et al. 2004; Negulescu et al. 1996; Valitutti et al. 1995; Miller et al. 2004a, 2004b).

When a firm contact is established, the T cell can give up its migratory morphology and round up again or remain stationary (Lee et al. 2003; Shakhar et al. 2005). However, it may also maintain a dynamic state of motility and crawl along the APC surface with substantial velocities of up to 10 $\mu\text{m}/\text{min}$ (Mempel et al. 2004; Gunzer et al. 2000, 2004; Miller et al. 2004a). How long will such individual contacts persist in vivo and where are they formed during an immune response? In the following sections we will have a closer look at the available experimental data and the models of T cell activation derived from them.

3.1

Classical In Vitro Studies of T Cell–APC Interaction

Initially, due to the absence of better technology, researchers were only able to take a static picture of the T cell–APC interaction. Classical experiments from Kupfer and Singer (1989) showed that antigen-specific T cells physically interacted with B cells presenting the relevant peptide antigen. Thus, the microtubule-organizing center (MTOC) of both cells became reoriented toward each other. It was the same group that 9 years later showed that upon interaction, the two cells formed a highly organized multimolecular assembly of proteins at the interaction plane that is now being referred to as the immunological synapse (IS) (Monks et al. 1998). At the same time it was demonstrated in liquid culture that naïve T cells need a certain period of time

in contact with APC to reach their full effector potential. This was ~ 6 h for the best possible APC, but could be as long as 30 h for weak APC. Therefore powerful APC, like DC, were able to induce T cell proliferation within a few hours of contact, even at low doses of antigen, while weak APC, such as B cells, needed long contact times, even at high levels of pMHC occupancy (Iezzi et al. 1998). In addition, the first approaches using live cell imaging of T cells migrating on lipid bilayers found that, in the absence of recognizable peptide–MHC complexes, T cells were highly mobile and migrated on ICAM-1 layers (Dustin et al. 1997). However, when a specific peptide–MHC complex was available within the lipid bilayers in addition to ICAM-1, the T cells stopped moving and remained at the MHC–peptide-containing spot (Dustin et al. 1997).

3.2

The Single Encounter Model of T Cell Activation

The above data were used to establish a model of T cell activation in lymph nodes stating that naïve T cells are actively motile in lymphoid tissues, thereby scanning the surface of all available APC for specific peptide–MHC complexes. However, as soon as a high-affinity (cognate) MHC–peptide complex is discovered, T cells stop and remain at this location, forming a stable cell contact with the APC for several hours until full activation is reached. During that time, an immunological synapse is formed, which is essential for signaling and which is possibly also involved in stopping T cell motility (Dustin and Chan 2000; Lanzavecchia et al. 1999). The concept of a cognate cell pair stopping and thus stabilizing its contact was appealing, as it provided a solution to the apparent problem that in vivo a rather low number of motile peptide-loaded APC had to activate an also very limited number of motile T cells (Nikolich-Zugich et al. 2004) specific for an invading pathogen. It appeared logical that, in the rare event of a specific APC hitting a specific T cell, this interaction had to be stabilized in order to maximize the chance of a successful T cell activation. Thus, the complete stopping of the two cellular partners was considered a general rule for all situations of specific T cell activation.

3.3

Challenges Introduced by Live Cell Imaging Experiments

This concept, however, was challenged by the first observations employing live cell imaging of antigen presentation in liquid cultures with T cell blasts and with macrophages serving as APC. Here, it was found that T cells migrate over APC bodies, stopping only for a limited amount of time on one APC, but then resume motility and wander on to the next APC. From these observations it was concluded that a T cell sequentially could gain signals from more

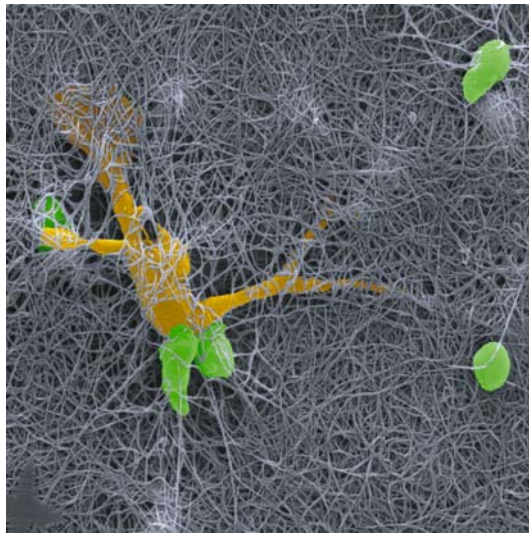


Fig. 4 T cells (*green*) interacting with an antigen-specific DC (*orange*) within 3D collagen. Note the huge size of the DC relative to the T cells, as well as the ability of the DC to “serve” three T cells at the same time. In vivo, DC can contact up to 200 cells at any given time point (Miller MJ et al. 2004a)

than one APC (Underhill et al. 1999). However, one point of critique regarding these experiments was that the particular behavior of the T cells used in this study could result from their preactivated state, and thus might not reflect the behavior of a naïve, nonactivated T cell. Therefore, in a new approach, naïve T cell receptor (TCR) transgenic T cells plus peptide-loaded highly activated DC were used as an antigen-specific activation pair, and the two types of cells were embedded in a 3D collagen matrix as an approximation of the 3D environment present within lymphatic tissue. Using this experimental system, it was shown that it was a general feature of naïve T cells to form contacts with specific antigen-loaded DC only transiently, for a few minutes rather than hours (Fig. 4) (Gunzer et al. 2000).

In addition, T cells were observed to engage multiple DC in a serial fashion. It was assumed that, although each of these contacts was able to induce signals within the T cell, none of these short-lived contacts was sufficient to activate the T cell but rather a sequence of several contacts was needed (Gunzer et al. 2000). It could later be shown that T cells may indeed gradually increase the level of signaling intermediates (Borovsky et al. 2002), and that the same level of activation can be reached by a sequence of interrupted contacts or with one stable contact (Faroudi et al. 2003). The intracellular events underlying these phenomena are not yet fully elucidated. Accumulation of transcription factors such as cJun over time following suboptimal TCR ligation has been demonstrated (Rosette et al. 2001); it was postulated that

a counting molecule exists, allowing for a stepwise buildup of activated signaling intermediates and finally leading to T cell activation (Rosette et al. 2001). The question as to whether such a system works like a capacitor, which “deloads” in one stroke once it has reached the loading capacity, or whether a more analog system exists, which can generate a smooth response reflecting different levels of activation, is currently only a matter of speculation.

3.4

A Serial Encounter Model of T Cell Activation

These findings were put together in the serial encounter model for T cell activation (Friedl and Gunzer 2001; Rachmilewitz and Lanzavecchia 2002). Based on the findings in 3D collagen matrices, the serial encounter model stated that there is no difference in the biophysical dynamics of T cells contacting low-affinity or cognate APC. However, only cognate APC were able to deliver a signal to the T cell. Therefore, the number of available pMHC complexes on one cell would not be essential, as it has been shown that even one pMHC molecule is sufficient to trigger T cell responses (Irvine et al. 2002). Since one contact was not enough, the T cell was forced to undergo additional contacts to other APC presenting the same antigen. Such a mechanism would be helpful in “measuring” the number of APC in the migratory reach of a T cell, and thereby inhibiting unnecessary immune activation in the case of minor infections with very low numbers of antigen-specific APC (Friedl and Gunzer 2001).

3.5

The Road to Intravital Imaging: Phase Models of T Cell Activation

The results of T cell biophysics obtained in 3D collagen matrices were challenged mainly by the fact that, in true lymphatic tissue, collagen fibers are not directly accessible to migrating cells. Rather, they are ensheathed by follicular reticulum cells of the lymphatic environment (Gretz et al. 2000; Kaldjian et al. 2001). Thus, the behavior of T cells in 3D collagen might not necessarily represent the *in vivo* reality (Dustin and de Fougères 2001; Dustin et al. 2001; Lanzavecchia and Sallusto (2001).

The goal to observe T cell activation in true lymphatic tissue was finally reached by two groups working with naïve T cells and DC in explanted lymph nodes (Miller et al. 2002; Stoll et al. 2002) and by a group examining thymocytes in reaggregate thymic organ cultures (Bousso et al. 2002). All three studies were able to demonstrate effective migration of T cells in lymphatic tissue. This was very remarkable given the extreme crowding of cells in these organs (Ushiki et al. 1995). In fact, for T and B cells, the measured motility parameters in intact lymphatic organs were identical to the values that were obtained for cells migrating in artificial 3D systems, which are only

sparsely populated such that individual cells only rarely touch each other (Gunzer et al. 2004; Friedl et al. 1994, 1995, 1998). Within T cell zones of lymph nodes DC, in contrast, were found to be more static. Rather than migrating within lymph nodes, they remained mainly stationary, but displayed highly motile dendrites which were constantly reaching for T cells (Miller et al. 2004a; Lindquist et al. 2004). Interestingly, the studies arrived at opposite conclusions concerning the duration of antigen-specific contacts between T cells and DC. While the studies by Bousso et al. (2002) and Miller et al. (2002) showed that contacts between T cells and APC were an equal mixture of short-lived and more extended interactions, Stoll et al. (2002) found exclusively long-lasting contacts between adoptively transferred mature DC and naïve T cells upon antigen presentation. It was possible that the differential results were due to differences in treatment of explanted tissues, or that changes in the behavior of cells after removal of the lymph nodes occurred. Therefore, it was clear that it was necessary to approach imaging in lymph nodes *in situ*, i.e., in the living animal.

This goal was initially reached by Miller et al. (2003), albeit only by looking at T cell migration. The first intravital study of naïve T cells interacting with DC in a lymph node was completed by Mempel et al. (2004). By monitoring CD8⁺ T cells, this study showed that the T cell activation followed a three-step mechanism. T cells which had just entered the lymph node engaged antigen-specific DC in short, transient encounters during the first 6–8 h. After this time, interactions became more stable (in the range of one or more hours) and the production of effector cytokines was induced. This phase lasted for ~ 12 h. A third stage was again characterized by profound motility and only transient encounters with DC. It was during this phase that T cell divisions became detectable (Mempel et al. 2004). These findings were essentially confirmed for CD4⁺ T cell activation *in vivo* (Shakhar et al. 2005) and in an explanted lymph node model (Miller et al. 2004b). In addition, the group of Amigorena (Hugues et al. 2004) showed that CD8⁺ T cells contacting DC under a regime inducing tolerance rather than activation displayed short-lived interactions at any time point, while Mempel et al.'s (2004) three-phase model with long interactions in a middle phase could be confirmed for an activatory situation (Hugues et al. 2004). Thus, current data suggest that a complete absence of static interactions to DC is associated with less efficient T cell activation or even T cell tolerization for CD8 cells, while there seems to be no obvious difference in interaction behavior of CD4 T cells, whether the outcome is anergy or activation (Shakhar et al. 2005). In any case, most available studies clearly show the presence of dynamic interactions at different time points during effective T cell stimulation.

The exact function and underlying molecular mechanisms of each respective phase are unclear; however, the sequence may reflect expression changes of surface receptors and cytoskeletal regulators during T cell activation. It is possible that the longevity of T cell–DC interactions in a phase might indicate

the formation of a mature synapse-like contact zone (Lee et al. 2002). While both dynamic and stable contacts contribute to T cell activation and proliferation, long-lasting interactions may be needed for maximum IL-2 production and the attainment of full effector functions (Hurez et al. 2003). The phase model or kinetic transition model of T cell interaction expands the serial encounter model by postulating an integration of several signals of different duration and strengths into a common pathway of T cell activation.

3.6

The Role of the APC

The results of recent studies lead to another important question. What cell makes the decision on maintaining the interaction and comigration? The T cell or the APC? The studies by Mempel et al. (2004) and Miller et al. (2004b) concluded that it was the status of the T cell which decided the duration of APC interaction. However, even in the rather homogeneous population of naïve TCR transgenic T cells, interactions with DC were shown to differ substantially *in vitro*, ranging from short-lived to long-lived (Hurez et al. 2003). The first study that addressed the role of the APC in deciding the duration of interaction used two types of effective APC—DC and activated B cells—as well as naïve B cells, as a model for a weak, ineffective APC. The APC were peptide-loaded and incubated separately with naïve TCR transgenic T cells. It was found that the T cells contacted effective APC (DC and activated B cells) in a short-lived manner, while they formed long-lasting contacts with inefficient APC (naïve B cells). This pattern was demonstrated both in 3D *in vitro* systems and in lymph nodes *in vivo* (Gunzer et al. 2004). The findings were even more unexpected as the long-lasting contacts with naïve B cells were shown to be the least efficient in terms of T cell activation, while the much more dynamic contacts to DC and activated B cells rapidly induced T cell activation (Gunzer et al. 2004).

3.7

Summary: A Multitude of T Cell–APC Interaction Kinetics

For the original problem of how to synchronize the migration of cells initially separated in space, live cell imaging experiments of the past 5 years have offered a wide range of solutions. Synchronization can occur transiently by limiting the migration of T cells to the surface of APC, either with an almost unchanged velocity compared to free migration or in a more stable manner, with strongly reduced motility down to complete stopping of T cells on the surface of DC. In a most extreme form, hour-long coordinated migration of a cell pair occurs, as in the example of naïve B cells as APC (Gunzer et al. 2004). During the comigration, very motile T cells were shown to push B cells *in vivo* through lymph node parenchyma, whereas B cells, when activated,

may take the lead themselves and pull T cells (Gunzer et al. 2004). The latter finding was later confirmed for B cells that were activated by loading antigen onto their B cell receptor (Okada et al. 2005). Another issue covered by the studies mentioned was directional migration. In the marketplace analogy, this would mean that customers on the market would somehow be preferentially attracted to or by specific booths. Currently available data do not support this idea. In contrast, there is evidence that migration of T cells in lymph nodes is entirely random and that encounters with APC are stochastic events (Miller et al. 2003; Wei et al. 2003). However, other researchers call these conclusions into question (Germain and Jenkins 2004). What is agreed upon is the sole fact that the lymph node per se is attractive to T cells, B cells, and APC by means of the chemokines CCL19 and CCL21 binding to the chemokine receptor CCR7 (Forster et al. 1999).

Lastly, it was shown that the character of interaction is not only modulated by antigenic load and type of APC, but also by the architecture of the surrounding tissue which may influence the dynamics of cell interactions. Tight dendritic cell networks within the lymph node facilitate *in vivo* T cell contacts with DC and the transition between them, thus allowing the T cells to be in contact with DC for most of the time (Lindquist et al. 2004; Westermann et al. 2005).

4

Conclusions and Outlook

Great advances in imaging techniques in recent years have led to unique insights into the complexity of cell–cell interactions *in vivo*. However, the resulting models of T cell activation still vary in their interpretation of physical interaction modes of T cells, and many aspects yet remain descriptive. There is now evidence from several research groups for a multistep cell interaction process during T cell activation. However, the molecular basis for the different interaction kinetics observed remains unclear. Other questions in need of clarification are, e.g., what is the relative importance of long over short contacts for T cell activation? Are both contact types necessary or is one dispensable *in vivo*? By what molecular principle do short cell contacts differ from long ones? Do short contacts “prepare” cells for long ones and if yes, how? How exactly is the surrounding tissue influencing the duration or outcome of interaction? Apart from studying effector cell induction, the mechanism of APC activation (especially of B cells) needs to be investigated. Experimental models need to be expanded to include locations other than inguinal or popliteal lymph nodes, e.g., lymphatic sites in the gut and the lung. More physiologically relevant models together with pathogen-related antigens are needed, as well as ways to overcome one major obstacle for *in vivo* imaging studies, the low number of precursor cells in wild-type animals.

In conclusion, T cells display an intriguingly complex pattern of cellular interaction with APC during their activation. A more complete understanding of the underlying mechanisms *in vivo* is needed to modulate or mimic such processes. This would be one prerequisite to make them amenable for therapeutic intervention. Based on the excellent advances in intravital imaging made in recent years, we can be very positive about future insights gained from this powerful methodology.

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Molecular Regulation of Cytoskeletal Rearrangements During T Cell Signalling

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Abstract Regulation of the cytoskeleton in cells of the haematopoietic system is essential for fulfilling diverse tasks such as migration towards a chemoattractant, phagocytosis or cell–cell communication. This is particularly true for the many types of T cells, which are at the foundation of the adaptive immune system in vertebrates. Deregulation of actin filament turnover is known to be involved in the development of severe immunodeficiencies or immunoproliferative diseases. Therefore, molecular dissection of signalling complexes and effector molecules, which leads to controlled cytoskeletal assembly, has been the focus of immunological research in the last decade.

In the past, cytoskeletal remodelling was frequently understood as the finish line of signalling, while today it becomes increasingly evident that actin and microtubule dynamics are required for proper signal transmission in many processes such as T cell activation. Significant effort is made in many laboratories to further elucidate the contribution of cytoskeletal remodelling to immune function.

The objective of this article is to summarise the current knowledge on how actin and microtubules are reorganised to support the formation of structures as diverse as the immunological synapse and peripheral protrusions during cell migration.

Keywords TCR · CD28 · Actin · Tubulin · Cytoskeleton · Immunological synapse

1 Introduction

Small GTPases act as molecular switches on which signalling inputs converge and are transduced into a coordinated array of output pathways leading to the desired cell response. The Ras family members, Ras and Rap1, have been assigned important functions for cytoskeletal reorganisation, particularly in integrin activation to initiate adhesion and migration (for review see Bos et al. 2003; Kinbara et al. 2003). Rho family GTPases, the best characterised of which comprise RhoA, Rac1 and Cdc42, regulate different sub-compartments of the actin cytoskeleton (reviewed by Hall 1998). While Rho activation triggers the generation of the contractile forces re-

quired, for instance, for force coupling through cell substrate adhesions, Rac and Cdc42 stimulate the protrusion of lamellipodia and filopodia, respectively (Nobes and Hall 1995). Moreover, Cdc42 appears to be crucial for cell polarisation in cells as diverse as budding yeast or cytotoxic T cells (Etienne-Manneville 2004b). In their activated state, these GTPases specifically interact with a plethora (> 80 known) of different effector proteins, some of which signal directly to actin cytoskeletal reorganisation. However, the exact functions of the various effector proteins in these processes are only just beginning to emerge.

1.1

The Actin Cytoskeleton

The highly dynamic and reversible polymerisation of actin is at the base of a number of complex cellular processes, such as chemotaxis or adhesion and cell-cell communication. Dysregulation of the actin cytoskeleton in haematopoietic cells has already been found to cause severe immunopathologies, such as Wiskott-Aldrich syndrome (Badour et al. 2004; Burns et al. 2004; Orange et al. 2004; Thrasher 2002) or BCR-Abl-induced leukaemia (Daley et al. 1990; Druker et al. 2001; Goldman and Melo 2003; Hantschel and Superti-Furga 2004; Pendergast and Witte 1987).

As a result of activatory signals, the polymerisation of actin filaments is initiated and maintained by nucleation and branching off from pre-existing filaments, processes thought to be catalysed by the Arp2/3 complex. Recently, proteins of the formin family were added to the list of cellular actin filament nucleators acting downstream of Rho-GTPases, and while the mechanism of creating long unbranched filaments was elucidated (Romero et al. 2004), the cellular function of the three diaphanous related formins (DRFs) and other formin family members in vertebrate cells is still far from being understood (Higgs 2005; Higgs and Pollard 2001; Zigmund 2004). The Arp2/3 complex consists of two actin-related and five additional subunits, and is essential for the protrusion of cellular projections such as lamellipodia (Machesky and Insall 1998; Pollard and Borisy 2003). The nucleating activity of the Arp2/3 complex can be stimulated, for instance, by proteins of the WASP/WAVE family of nucleation promotion factors (NPFs), which all share a unique C-terminal domain designed to efficiently activate the Arp2/3 complex (Millard et al. 2004; Stradal et al. 2004). The protein regions outside this module, however, define two subfamilies: in mammals, one subfamily comprises the haematopoietic WASP (Wiskott-Aldrich syndrome protein) and the more ubiquitously expressed N-WASP (neural WASP), while the second subfamily is built of three WAVEs (WASP family verprolin-homologous proteins), also known as Scar (suppressor of cAMP receptor) isoforms.

Differential roles for both protein subfamilies, WASP/N-WASP and WAVES, will be discussed below with a focus on their potential as well as established roles in the spatio-temporal activation of T cells.

1.2

The Microtubule System

Microtubules are involved in cell division, cell motility, morphogenesis and cell polarity, translocalisation of organelles and transport of vesicles (Etienne-Manneville 2004a; Sancho et al. 2002; Vyas et al. 2002). T lymphocytes are highly motile premitotic cells harbouring a well-developed microtubule cytoskeleton (see Fig. 1) upon activation they have the ability to migrate, to form intimate cell–cell contacts and finally to enter the cell cycle and undergo mitosis. The microtubule building block is formed by heterodimers of α - and β -tubulins, and head-to-tail association of these dimers leads to the formation of linear protofilaments. The lateral association of at least 13 protofilaments forms a cylindrical microtubule. Microtubules are inherent polar structures with a dynamic plus end and a minus end that can be stabilised by embedding the filament into a microtubule organising centre (MTOC). The dynamic behaviour of microtubules *in vivo* is described by the term dynamic instability, as a microtubule can frequently switch between phases of growth and rapid depolymerisation. Post-translational enzymatic modifications of tubulin, which can occur after polymerisation into tubules, lead to variations in microtubule stability and altered affinity for microtubule binding proteins (MacRae 1997; Westermann and Weber 2003).

Dynamic microtubules can be locally stabilised during cell migration and polarisation (Gundersen et al. 2004). Microtubule stabilisation in fibroblasts occurs primarily at the leading edge and results from capturing of the microtubule plus-ends by plasma membrane–microtubule tip complex interactions (Brunner 2002; Gundersen and Cook 1999; Gundersen et al. 2004). These stabilised microtubules accumulate post-translationally modified tubulin, such as acetylated or deetyrosinated tubulin (for reviews see MacRae 1997; Westermann and Weber 2003). Acetylation of α -tubulins occurs on a conserved lysine residue at position 40 at the amino terminus of α -tubulins (LeDizet and Piperno 1987; L'Hernault and Rosenbaum 1985). To date, the enzymes that are responsible for the tubulin acetylation in mammalian cells remain elusive (Polevoda and Sherman 2002). The enzymes that catalyse the opposing reaction, deacetylation, have been described. HDAC6 (histone deacetylase 6), a member of the histone deacetylase family, deacetylate tubulin and microtubules *in vitro*, and suppression of HDAC6 by pharmacological inhibitors (trichostatin (TSA) and tubacin) or by small interfering RNA (siRNA) leads to a marked increase in tubulin acetylation in fibroblasts (Haggarty et al. 2003; Hubbert et al. 2002; Zhang et al. 2003).

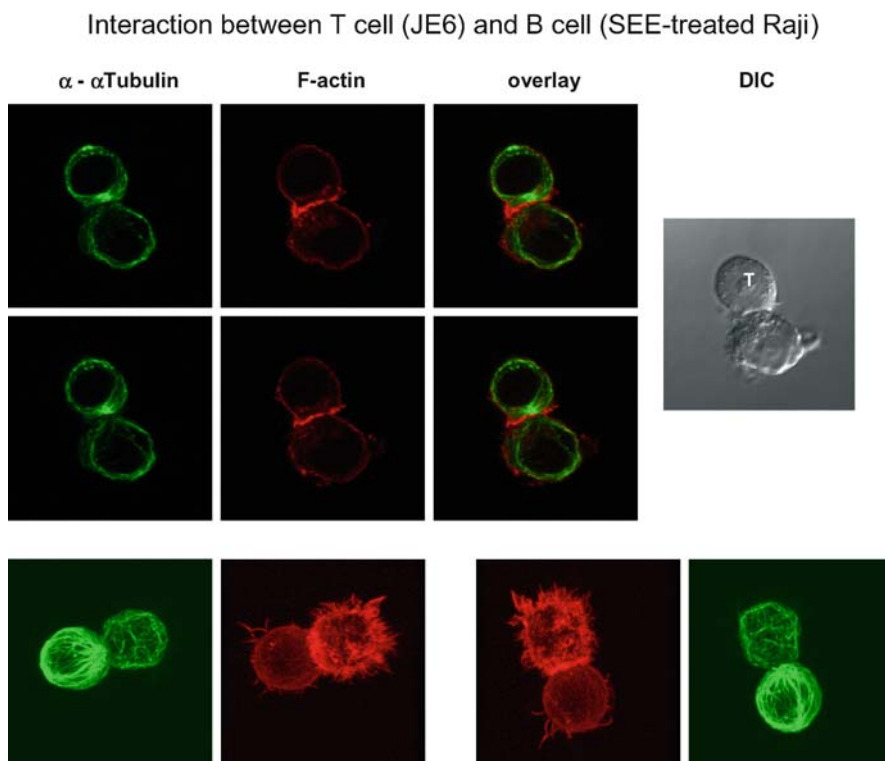


Fig. 1 Visualisation of MTOC polarisation and the actin cytoskeleton after conjugation of a T cell with a B cell. B cells were loaded with the superantigen SEE and conjugated with T cells. F-actin was visualised using TRITC-phalloidin (*red*) and microtubules were detected with an antibody against α -tubulin in combination with an anti-mouse IgG FITC (*green*). The *upper panels* show two different confocal sections through the same T cell/APC conjugate, as also shown in the differential interference contrast (DIC) image on the *right*. The *lower panel* displays two different 3D reconstructions of the same T/B cell couples

The best characterised post-translational modification of α -tubulins, however, is the tyrosination cycle, which involves the reversible removal of the carboxy-terminal tyrosine residue from α -tubulin by a so far uncharacterised tubulin carboxypeptidase and the re-addition of a tyrosine residue by an enzyme termed tubulin tyrosine ligase (TTL) (Erck et al. 2000). The tyrosination cycle generates two tubulin pools: intact tyrosinated α -tubulins (Tyr-tubulin) and detyrosinated α -tubulins, which lack the C-terminal tyrosine exposing a glutamic acid as the new C terminus (Glu-tubulin). Glu-tubulin is enriched in stable microtubules, which exhibit little dynamic behaviour, whereas dynamic microtubules display mostly Tyr-tubulin. Recently, the importance of the tyrosination cycle has been shown by generating a TTL knockout mouse. These mice died shortly after birth due

to disorganisation of the neuronal networks (Erck et al. 2005). Below, the involvement of microtubule dynamics and modifications in cell polarisation, during cell–cell interaction or cell migration in T lymphocytes will be discussed.

2

Arp2/3-Dependent Actin Assembly and the WASP/Scar Family of Proteins

2.1

WASP and the Wiskott–Aldrich Syndrome

In mammals, most is known about the loss of function of the haematopoietic WAS gene. In addition to the intense research on the inherited Wiskott–Aldrich syndrome (WAS), two independent WASP knockout mice have been reported (Snapper et al. 1998; Zhang et al. 1999), displaying a mild haematopoietic phenotype reminiscent of less severe WAS in humans (Burns et al. 2004). In 1996, 2 years after the discovery of WASP as the gene mutated in this disease (Derry et al. 1994), two groups independently established the direct interaction of this protein with the Rho family GTPase Cdc42 (Aspenstrom et al. 1996; Symons et al. 1996), raising the possibility of WASP acting in the translation of Cdc42 signalling to actin cytoskeletal reorganisation. The subsequent recognition of the Arp2/3-activating features of WASP/Scar family members (Machesky and Insall 1998; Machesky et al. 1999; Rohatgi et al. 1999) provided the direct connection to actin filament assembly. Hence, a potential $\text{Cdc42} \rightarrow \text{WASP/N-WASP} \rightarrow \text{Arp2/3}$ pathway appeared firmly established and provided a first clue on how Cdc42 could induce actin assembly. The most striking cytoskeletal phenotypes in WAS patients and WASP knockout mice are impaired T cell activation upon T cell/antigen-presenting cell (APC) conjugation (Gallego et al. 1997), the lack of surface microvilli on T lymphocytes (Kenney et al. 1986) (although this was questioned recently (Majstoravich et al. 2004)), as well as the failure of haematopoietic cells to form podosomes or to efficiently migrate in vitro (Jones et al. 2002). Indeed, both WASP and N-WASP localise to podosomes and appear essential for their formation (Mizutani et al. 2002).

2.1.1

Regulation of WASP

The activity of the ubiquitously expressed N-WASP is regulated through a plethora of binding partners such as Cdc42 (Rohatgi et al. 1999), PIP2 (phosphatidylinositol 4,5-bisphosphate) (Rohatgi et al. 2000), WASP-interacting protein (WIP) (Moreau et al. 2000), and SH3 domain harbouring

signalling adapters like Nck (Rohatgi et al. 2001) or Grb2 (Carlier et al. 2000). Thus, these signalling intermediates are thought to play key roles in the regulation of actin rearrangements, providing the diversity necessary to satisfy the various requirements. Although many interaction partners of N-WASP are known, most of them were implicated in N-WASP activation or modulation of its activity. Until recently, it was widely agreed that N-WASP, which is autoinhibited in the cytoplasm via an intramolecular bond, exists as a monomer prior to activation. Subsequently, however, biochemical approaches indicated that N-WASP appears constitutively bound to WIP family members, such as the brain-specific CR16 (Ho et al. 2001), or to WIP (= WASPIP) (Ho et al. 2004), which is highly expressed in the haematopoietic system (Anton et al. 2002). Furthermore, Ho and colleagues (2004) concluded from *in vitro* actin polymerisation assays that WIP binding to N-WASP stabilises its inactive conformation. However, other studies suggest a dependency on the binding of WIP to the N terminus of WASP during T cell activation (Silvin et al. 2001), and a positive cooperation between WIP and WASP/N-WASP has also been described in other systems (Martinez-Quiles et al. 2001; Moreau et al. 2000). Moreover, WIP-deficient T cells display a phenotype significantly reminiscent of WASP deficiency, as exemplified by impaired proliferation, reduced interleukin-2 (IL-2) secretion or disturbed immunological synapse (IS) formation after T cell receptor (TCR) ligation (Anton et al. 2002). Thus, it is still controversial as to whether the *in vitro* findings of Ho and colleagues (2004) describing WIP as an inhibitor of WASP/N-WASP function can be confirmed *in vivo*.

Beyond WIP, only little is known about the direct interaction partners of WASP as compared to N-WASP. In most cases, newly identified interaction partners of N-WASP are assumed to interact with WASP in a similar manner, although this was rarely tested. One exception is the finding that the Ena/VASP family member, VASP (vasodilator-stimulated phosphoprotein), interacts with WASP but not N-WASP and promotes actin assembly at the plasma membrane (Castellano et al. 2001). A WASP/VASP-containing complex was reported to occur in macrophages during FC γ -receptor-mediated phagocytosis (Coppolino et al. 2001), and sequestration of Ena/VASP proteins in Jurkat T cells using a fragment of the listerial virulence factor ActA (Sechi et al. 2002) was found to impede full interaction with anti-CD3-coated beads. The relevance of these interactions *in vivo* remains to be established.

2.1.2

WASP and Endocytosis

WASP is implicated in the clathrin-mediated endocytosis of the T cell antigen receptor (McGavin et al. 2001; Zhang et al. 1999), and both WASP and N-WASP are known to interact with various endocytic proteins, e.g.

syndapin (also named PACSIN) proteins (reviewed by Kessels and Qualmann 2004), intersectin-1 (Hussain et al. 2001) and indirectly with the large GTPase dynamin (reviewed by Engqvist-Goldstein and Drubin 2003; Schafer 2002). Hence, WASP and N-WASP are thought to play a role in receptor-mediated endocytosis (Gundelfinger et al. 2003; Qualmann and Kessels 2002).

In exciting recent studies, direct visualisation of the recruitment of actin and N-WASP accompanying the internalisation of single clathrin-coated pits was achieved by employing two-colour total internal reflection fluorescence (TIRF) microscopy (Merrifield et al. 2002, 2004). Moreover, endocytic defects were observed in cells ablated for N-WASP expression by genetic deletion as well as by RNA interference. In N-WASP null fibroblasts, impaired actin assembly at internalising clathrin-coated pits was accompanied by a significant reduction in internalisation rates of epidermal growth factor receptor (EGFR) (Benesch et al. 2005), and after RNAi-mediated knockdown of N-WASP additional severe alterations in cell surface distribution of EGFR and transferrin receptor (TfR) were observed in HeLa cells (Innocenti et al. 2005).

Moreover, several lines of evidence substantiate a physiological function of N-WASP/WASP in actin assembly events at the plasma membrane or endomembranes. For instance, N-WASP $-/-$ fibroblasts fail to support the movement of endosomal vesicles evoked by increased PIP2 or phosphotyrosine levels (Benesch et al. 2002; Taunton 2001), a phenotype which can be fully restored not only by the re-expression of N-WASP but also by expression of WASP. Thus, both the haematopoietic WASP and N-WASP may drive actin assemblies for complex cellular functions related to membrane and protein traffic, which require further analysis.

2.2

WAVE Proteins Induce Actin Assembly Downstream of Rac

All haematopoietic cells express members of the WAVE subfamily of proteins in addition to WASP. WAVE proteins mediate Arp2/3-driven lamellipodia and membrane ruffle formation (Machesky and Insall 1998; Miki et al. 1998), which occur concomitant to Rac1 activation. However, unlike WASP or N-WASP, WAVE proteins lack a motif capable of direct interaction with a Rho-GTPase, and instead are linked to Rac1 indirectly.

Several studies have established how WAVE2 function is controlled by a multi-protein complex consisting of at least four components, which links Rac to Arp2/3 activation (Eden et al. 2002; Innocenti et al. 2004; Kunda et al. 2003; Rogers et al. 2003; Steffen et al. 2004). This complex harbours the specifically Rac1-associated protein 1 (Sra-1) and Nck-associated protein 1 (Nap1), Abi (Abl interacting) proteins and WAVE. The constitutively assembled complex was found to be recruited to and activated at the plasma membrane upon activation of Rac1. However, the role of the WAVE complex in the formation of

structures other than lamellipodia or in cells other than fibroblasts remained elusive (see also below).

Mammals express three WAVE isoforms, two of which are mostly restricted to the brain (WAVE1 and WAVE3) and one (WAVE2) being expressed ubiquitously (Sossey-Alaoui et al. 2003). Conventional WAVE2 knockout mice have not helped the understanding of WAVE function in haematopoietic cells due to early embryonic lethality (Yamazaki et al. 2003; Yan et al. 2003). It will greatly improve our understanding of WAVE complex function in the immune system when the results from conditional targeting of WAVE genes, and subsequent deletion of proteins in cells of the haematopoietic lineage, become available.

3

T Cell Signalling Leading to Cytoskeletal Rearrangements

The Rho family of GTPases is most prominently involved in the regulation of cytoskeletal rearrangements. We will now briefly summarise two prominent T cell behaviours, cell migration along chemotactic gradients and activation of TCR/CD3 complexes through interaction with an APC, which both involve signalling cascades leading to activation of GTPases (Cantrell 2003) and subsequent rearrangement of the actin and microtubule cytoskeleton.

3.1

T Cell Migration

Haematopoietic cells including T cells display a type of movement that has been termed amoeboid movement, which differs significantly in phenotypic appearance from fibroblastic migratory behaviour (Entschladen and Zanker 2000). This amoeboid type of migration (Fig. 2) was repeatedly compared to the chemotactic movements of *Dictyostelium discoideum* (Parent 2004; Van Haastert and Devreotes 2004). Directional cell migration entails the establishment and maintenance of polarity, as established by formation of protrusive actin-based structures at the cell front (Pollard and Borisy 2003; Small et al. 2002). This allows the exploration of new space and is accompanied by anchorage of these newly formed cellular structures through specialised adhesion sites, which then support dragging of the cell body forward (Beningo and Wang 2002; Hestermann et al. 2002; Horwitz and Parsons 1999) and—in most cell types—reorientation of the microtubule cytoskeleton (Etienne-Manneville and Hall 2003; Gundersen 2002; Palazzo et al. 2001). For immune cells, and also for the well-studied *D. discoideum*, chemotactic movement is initiated when chemoattractants bind to seven-membrane-span receptors that couple to heterotrimeric G proteins. This leads to activation of a plethora of effector and adaptor proteins through the dissociated $G\alpha$ and $\beta\gamma$ subunits

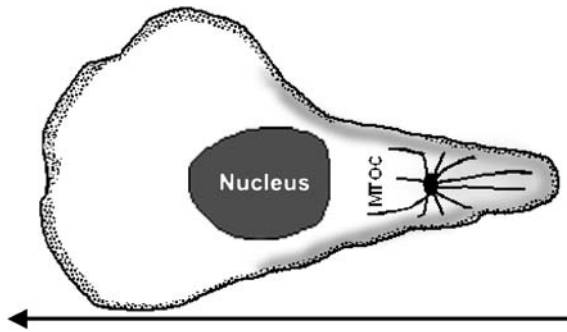


Fig. 2 Schematic representation of a migrating T lymphocyte. The leading front of the cell displays F-actin-rich lamellipodia-like protrusions called pseudopodia; the trailing edge of the cell is termed the uropod and is enriched in myosin II. In contrast to most other cell types, the MTOC of migrating T cells is packed into the uropod and it is unknown whether or how the MTOC or microtubules influence T cell movement during chemotaxis

(Parent 2004), ultimately leading to cellular polarisation and movement. Consequently, pre-treatment of Jurkat T cells with pertussis toxin, a $G_{\alpha i}$ inhibitor, abolishes chemotaxis towards SDF-1 α (Okabe et al. 2002). In comparison to other cell types, T lymphocytes are the smallest and fastest migrating cells (Entschladen et al. 1998; Miller et al. 2003; Nikolai et al. 1998; Smith et al. 2003).

The chemokine SDF-1 α (denotes stromal cell-derived factor-1 α , also termed CXCL12), originally characterised as a pre-B cell stimulatory factor, is a potent chemoattractant for T lymphocytes and other leukocytes (Kim and Broxmeyer 1999). Targeted disruption of the SDF-1 gene (or its unique receptor CXCR4) in mice is perinatally lethal with defects in lymphopoiesis and bone marrow myelopoiesis (Nagasawa et al. 1996). Little is known about the signalling pathways that mediate SDF-1 effects. SDF-1 α stimulation enhances the tyrosine phosphorylation of various focal adhesion molecules, such as Crk and paxillin and, most importantly, T lymphocyte chemotaxis requires phosphatidylinositol (PI) 3-kinase activity and is abrogated after chemical inhibition of this enzyme with either wortmannin or Ly294002. (Dutt et al. 1998; Okabe et al. 2002; Wang et al. 2000). Data from *D. discoideum* underscore the fact that recruitment of PI 3-kinase and localised PIP3 production at the leading front of chemotaxing cells are upstream of pseudopod protrusion, as also occurs in the presence of the actin polymerisation inhibitor latrunculin (Janetopoulos et al. 2004).

But where in chemotaxis do WASP- and WAVE-mediated Arp2/3 activation come into play? Acquisition of polarity is at the onset of directed migration and accompanied by drastic redistribution of cytoskeletal components, during which F-actin and numerous actin-binding proteins are enriched at the front or leading edge and myosin II is assembled on the sides and at the back

or trailing edge, also termed uropod (Entschladen and Zanker 2000; Hogg et al. 2003).

WASP-deficient monocytes display impaired chemotaxis in response to monocyte chemoattractant protein-1 (MCP-1) (Badolato et al. 1998) and Fc γ receptor-mediated phagocytosis is inhibited (Lorenzi et al. 2000). T lymphocytes from WAS patients display abnormal chemotaxis in response to the T cell chemoattractant SDF-1 α (Haddad et al. 2001). In this study Cdc42 was shown to be crucial for the chemotactic response, and thus maybe for activation of WASP, by sequestering specifically activated Cdc42 using the recombinant Cdc42 interactive motif (CRIB domain) of WASP (Haddad et al. 2001). WASP was tyrosine phosphorylated in response to SDF-1 α stimulation in Jurkat T cells, a phenomenon that was described earlier to augment WASP activation (Cory et al. 2002, 2003). Moreover, SDF-1 α induced Crk-associated substrate (Cas), Nck, and focal adhesion kinase (FAK) phosphorylation in Jurkat T cells in response to SDF-1 α . Consequently, chemotaxis in these cells can be inhibited using herbimycin, a protein tyrosine kinase inhibitor. Notably, as opposed to defective chemotaxis, the random motility of, for example, WAS patient macrophages was found to be indistinguishable from that of control cells (Jones et al. 2002; Zicha et al. 1998), suggesting that WASP deficiency does not interfere with the formation of cellular protrusions, but rather with the establishment and/or maintenance of polarity towards the chemotactic stimulus.

Data from the chemotaxis of *Dictyostelium* support this view: *Dictyostelium* harbours two copies of the WASP gene and one for Scar (= WAVE). A *Dictyostelium* strain was designed that expresses very low levels of WASP after genetic deletion of one gene and tetracycline-dependent suppression of the expression of the other gene (Myers et al. 2005). Cells of this strain were more spread and flattened as compared to wild-type (WT) cells, but were unable to establish polarity resulting in a higher angular deviation and thus a four times lower speed of movement towards the chemoattractant (Myers et al. 2005). In contrast, Scar null *D. discoideum* cells (Bear et al. 1998; Blagg et al. 2003) were less well spread and hence significantly smaller than WT cells. They displayed strongly reduced F-actin levels and were unable to accumulate F-actin at the leading front during chemotaxis, although they were able to polarise within the chemotactic gradient (Bear et al. 1998). So both deficiencies, WASP (Myers et al. 2005) and Scar (Bear et al. 1998; Blagg et al. 2003), were significantly impaired in chemotaxis and showed reduced speed and total F-actin levels, but while in WASP-deficient cells polarisation was abrogated, in Scar null cells pseudopod formation was defective.

One striking difference that has been reported between migrating T cells and other cell types, including fibroblasts and *D. discoideum*, is that the MTOC becomes packed into the uropod of T cells (Entschladen et al. 2000; Ratner et al. 1997), while in, for example, *Dictyostelium* the MTOC is located in front of the nucleus, heading and reorienting in the direction of the leading

front pseudopods (Ueda et al. 1997). So far, no direct involvement of MTOC polarisation in T cell migration has been found. It is noteworthy that depolymerisation of microtubules in T cells even leads to an increase in locomotory activity (Nikolai et al. 1998), thus supporting the hypothesis that they do not utilise microtubule–focal contact interactions as described, for example, for fibroblasts, in the process of migration.

A role for tubulin acetylation in cell motility has been proposed on the basis that HDAC6 overexpression increased the chemotactic movement of NIH3T3 cells, whereas inhibition of HDAC6 blocks cell migration (Haggarty et al. 2003; Hubbert et al. 2002).

The microtubule network is also associated with a growing number of signalling molecules that are probably involved in T cell migration. In T cells, paxillin localises not only to areas of LFA-1/ICAM-1 and VLA-4/fibronectin attachment sites, but also to the MTOC and direct binding of paxillin to γ -tubulin was described (Herrerros et al. 2000; Sancho et al. 2000). LFA-1 engagement promotes translocalisation of Pyk-2 (Rodriguez-Fernandez et al. 1999), PKC β I and PKC δ (Volkov et al. 1998) to the MTOC of the migrating cell. PKC β I also co-localises with microtubules, and LFA-1 has been isolated as part of a PKC β I-tubulin-rich complex in T cells, which is thought to be important for LFA-1-mediated locomotion of activated T cells (Volkov et al. 1998, 2001). For a more detailed discussion on the important role of adhesion in chemotaxis beyond the described MTOC interactions of adhesion molecules, the reader is directed to recent excellent reviews on the subject (DeMali et al. 2003; Kinbara et al. 2003; Laudanna et al. 2002).

3.2

Immunological Synapse Formation/TCR Signalling

Formation of the IS is initiated by T cell receptor (TCR) recognition of a peptide/MHC (major histocompatibility complex) complex. This interaction induces a tyrosine phosphorylation cascade and is first seen as a ring surrounding a central cluster of LFA-1 (leukocyte function-associated molecule 1), which later inverts to give rise to the mature appearance of the IS (see Fig. 3a and b).

3.2.1

TCR Activation

Ligand recognition causes the T cell to stop migrating and to form a stable cell–cell contact with the corresponding APC, termed immunological synapse (Peacock and Jirik 1999). T cell–APC contact formation involves reorientation of the MTOC (including associated vesicles and Golgi), and the recruitment of receptors and signalling molecules to the nascent immunological synapse (for schematic overview see Fig. 3b) (reviewed by Huppa and Davis 2003). When

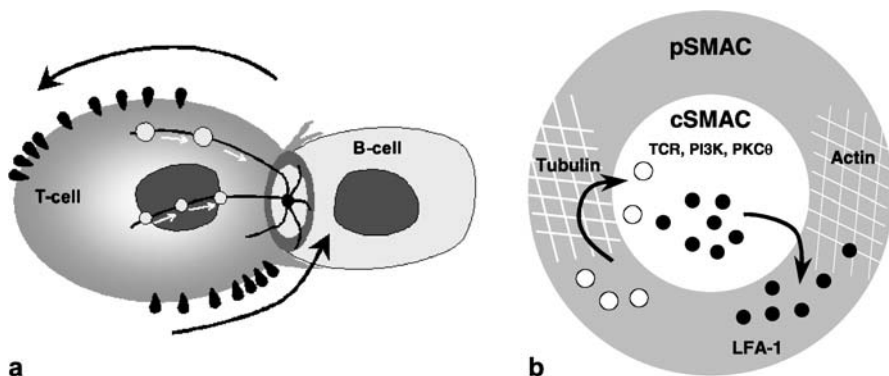


Fig. 3 **a** Schematic overview of a T cell building up contact with a B cell to form the immunological synapse (IS). The contact area between the two cells forms supramolecular activation clusters (SMACs) with at least two distinct areas: the peripheral p-SMAC and the central c-SMAC, which contain defined sets of proteins. Some T cell surface molecules become excluded from the zone of cell–cell contact during synapse formation (*upper arrow*), whereas others become recruited into the IS. The T cell MTOC is located in the ultimate vicinity of the TCR-containing c-SMAC in the mature synapse, and secretory vesicles become transported to this site in a microtubule-based fashion. Depending on the T cell type, they contain stimulatory cytokines (like IL-2 in CD4⁺ T cells) or cytolytic agents (such as granzyme or perforin) in CD8⁺ T cells. **b** Front view of the SMACs in a mature IS. The cell–cell contact region is cholesterol enriched (raft-type membrane). In the c-SMAC the TCR, phosphatidylinositol 3-kinase (PI3K) and protein kinase C theta (PKC θ) are enriched and active, while adhesion molecules such as the integrin LFA-1 can be found in the outer p-SMAC. Accumulating evidence suggests that WASP-based actin polymerisation and focal adhesion like F-actin anchorage, as well as membrane–microtubule anchorage, occur predominantly in the peripheral p-SMAC zone

a T cell encounters an APC carrying the appropriate MHC/peptide complex, within seconds a tyrosine phosphorylation cascade is initiated starting from the engaged TCRs. Phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3/zeta complex through the src-kinases Lck and Fyn creates the docking sites for zeta-associated protein of 70 kDa (ZAP-70), which in turn binds to the ITAMs via its tandem Src homology 2 (SH2) domain, thereby placing ZAP-70 in proximity to Lck. Lck phosphorylates and activates ZAP-70, which in turn phosphorylates T cell signalling components including the transmembrane adaptor protein linker for activating T cells (LAT) (reviewed by Kliche et al. 2004; Togni et al. 2004; Horejsi et al. 2004) and SH2-domain-containing leukocyte protein of 76 kDa (SLP-76). As a consequence of phosphorylation, LAT and SLP-76 recruit other adaptor proteins including Grb2, Gads as well as phospholipase C γ 1, the guanine nucleotide exchange factor (GEF) Vav1 and the Tec kinase Itk (Horejsi et al. 2004; Lucas et al. 2003). In this stage of T cell activation, the molecules are being translocated into the contact area between both cells and, in addition to tyrosine

phosphorylation, a first upsurge of actin polymerisation can be detected. Inhibition of both src-kinases by PP2 or in Lck-deficient Jurkat T cells (Badour et al. 2004; Bromley et al. 2001a) or actin assembly (Valitutti et al. 1995) interrupts T cell activation. This early time point of TCR signalling is believed to be the one where activation of small GTPases through various GEFs, such as Vav1, occurs (Tybulewicz et al. 2003), which is required for formation of the actin-based structures. Within the next 15–20 min, all molecules in the nascent immunological synapse become organised in so-called supramolecular activation clusters (SMACs) with a central (c-SMAC) and a peripheral (p-SMAC) zone, as described in great detail in excellent recent reviews (Bromley et al. 2001a; Friedl and Gunzer 2001; Friedl and Storim 2004; Kupfer and Kupfer 2003).

MTOC Polarisation During T Cell–APC Conjugation

T cells, upon engaging to an APC, reorient their MTOCs together with associated organelles such as the Golgi apparatus, granules and vesicles towards the target cell (Friedl and Storim 2004). Recent studies showed that this re-orientation was required for both cytotoxic T lymphocyte (CTL)-mediated cytotoxicity and T helper functions (Miletic et al. 2003; Poenie et al. 2004; Sancho et al. 2000; Vyas et al. 2002). In activated T helper (CD4+ T cells), site-directed secretion of cytokines such as IL-2 and IL-4 is accompanied by and seems to depend on translocalisation of the MTOC (Kupfer et al. 1991, 1994; Reichert et al. 2001), as also exemplified by the polarised secretion of cytokines after experimentally induced TCR cross-linking (Poo et al. 1988). One to five minutes after the initial contact with the B cell, the MTOC of the T cell is moving towards the site of cell–cell contact (Kuhne et al. 2003; Tskvitaria-Fuller et al. 2003). The signals exchanged by interaction of T cell–B cell pairs subsequently lead to activation, gene transcription, proliferation and differentiation within both cells (Bromley et al. 2001b; Gunzer et al. 2004). Furthermore, when multiple B cells were attached to the T helper cells, the B cells next to the MTOC-polarised Golgi apparatus were stimulated to proliferate (Gunzer et al. 2000; Reichert et al. 2001; Depoil et al. 2005).

Recently, Serrador and co-workers showed that TCR engagement enhances the formation of acetylated tubulin. Acetylated microtubules were concentrated at the T cell–B cell contact site and were surrounded by the TCR complex and LFA-1. Overexpression of HDAC6 but not a kinase dead HDAC6 mutant disorganised the TCR complex and LFA-1 at the immunological synapse. In addition, MTOC translocation towards the contact site and IL-2 production were impaired in HDAC6-overexpressing T cells (Serrador et al. 2004). These results suggested that a subset of acetylated microtubules is necessary for polarisation of the MTOC and stabilisation of the immunological synapse, and influences T cell activation. It will be interesting to see if either Vav1, SLP-76, ZAP-70 or LAT, which have been shown to regulate MTOC

polarisation to the contact site, are modulating the formation of acetylated microtubules.

In cytotoxic T cells, reorientation of the MTOC allows the Golgi apparatus and lytic granules to be deposited into the contact site (Kupfer et al. 1985; Stinchcombe et al. 2001). The function of MTOC polarisation in cytotoxic T cells is to direct effector activities to a specific target cell by confining the delivery of lytic granules and cytokines to a small restricted area of contact, an event that takes place within 5 min after target cell encounter (Stinchcombe et al. 2001). The inability of non-lytic tumor-infiltrating lymphocytes to polarise the MTOC was described to preclude translocalisation of lytic granules and to prevent target lysis (Radoja et al. 2001). Using modulated polarisation microscopy to study MTOC translocalisation in living cytotoxic T cells, Kuhn and colleagues (Kuhn and Poenie 2002; Kuhn et al. 2001) showed that the MTOC follows a straight line as it moves towards the synapse. Upon reaching the synapse, the MTOC begins to oscillate slightly along the contact zone. The oscillating movements of the MTOC suggest that cortical dynein anchored at the synapse probably generates tension on microtubules. Analysis of the 3D array of microtubules in CTL target pairs showed that microtubules anchored to the same regions of the contact site as that where the integrin LFA-1 was clustered in the p-SMAC. Furthermore, in cytotoxic T cells interacting simultaneously with two susceptible target cells, the MTOC was seen to oscillate between the targets in a straight line, suggesting that the MTOC retained its connection with both target cells while cytolysis probably occurs sequentially (Kuhn and Poenie 2002).

Recent studies have shed more light on the signalling pathways required for MTOC polarisation. Repositioning of the MTOC is a complex event and depends on engagement of the TCR but not on CD28 or the integrin LFA-1 (Kuhne et al. 2003; Lowin-Kropf et al. 1998; Sedwick et al. 1999). The TCR dependence of MTOC polarisation required Lck and subsequent ZAP-70 activation and the presence of phosphorylated ITAMs (Lowin-Kropf et al. 1998; Sedwick et al. 1999). Using Jurkat T cell lines deficient for ZAP-70, Lck, LAT and SLP-76, several groups could show that these signalling molecules are required for MTOC polarisation (Blanchard et al. 2002; Kuhne et al. 2003; Sedwick et al. 1999). The adaptor protein SLP-76 is linked to the actin cytoskeleton by its ability to interact with Vav1, Cdc42, Nck and the Wiskott–Aldrich syndrome protein (WASP) (Krawczyk et al. 2002; Zeng et al. 2003). Moreover, a report in which Tian and colleagues (2000) described the fact that the Cdc42 effector CIP4 can recruit WASP to microtubules suggests that the actin cytoskeleton and MTOC repositioning may be intimately connected. Clearly more research is needed to further characterise the signalling components involved in MTOC polarisation and function.

Finally, accumulating evidence suggests that TCR-mediated signalling peaks before the mature synapse is formed (reviewed by Davis and Dustin 2004; Lee et al. 2002), and this is when actin reorganisations as well as MTOC

reorientation are initiated. Hence, models are questioned in which the duration of T cell–B cell interaction (> 12 h) was hypothesised to be required for sustained TCR signalling. In elegant studies and using T cells from mice genetically deficient for CD2ap (CD2-associated protein), Lee and colleagues (2002, 2003) showed that extended TCR signalling even impairs formation of a mature IS.

3.2.2

Co-Stimulation Through CD28: A Matter of Cell Fate

Full activation of T cells results in proliferation, which allows clonal expansion specific for the antigenic determinants causative of T cell/APC recognition. This requires not only TCR-proximal signalling but also co-stimulation through CD28 with CD80 or CD86 (Acuto and Michel 2003; Rudd and Schneider 2003). CD28-deficient mice or mice treated with antagonists of CD28–CD80/CD86 interaction display reduced responses to an array of immune challenges, including infectious pathogens (Compton and Farrell 2002; King et al. 1996; Mittrucker et al. 2001; Shahinian et al. 1993), allograft antigens (Salomon and Bluestone 2001), graft versus host disease (Via et al. 1996), contact hypersensitivity (Krinzman et al. 1996) and asthma (Kondo et al. 1996). Correspondingly, the lack of CD28-mediated co-stimulation of T cells results in impaired proliferation *in vitro* and *in vivo*, reduced T helper cell differentiation (Gudmundsdottir et al. 1999; Lucas et al. 1995) and reduced expression of Th2-type cytokines (Rulifson et al. 1997). In summary, animal and cell-based studies have confirmed that a lack of interaction of CD28 with its natural ligands attenuates the T cell activation cascade (Lenschow et al. 1996).

In humans, T cells lacking detectable expression of CD28 are the most reliable biological indicator of senescence in the immune system and are symptomatic for age-related immunoincompetence (reviewed by Vallejo 2005). While CD28 plays a crucial role in enhancing cytokine production for the differentiation and survival of T cells, as well as control of the cell cycle (for review see, e.g., Acuto and Michel 2003; Bluestone 1995), little is known about its relationship to actin cytoskeleton remodelling. Stimulation of CD28 in the absence of TCR stimulation leads to its tyrosine phosphorylation (Tsuchida et al. 1999) and subsequent recruitment PI3K, Vav1, Itk or Grb2 (Hehner et al. 2000; Kim et al. 1998; Pages et al. 1994; August et al. 1994; Michel et al. 2001; Yang et al. 1999). Nevertheless, no specific roles for CD28-dependent actin remodelling were described, hence leading to a model in which CD28 (and related co-stimulatory or co-inhibitory receptors) controls priming, differentiation and maturation of the T cell response rather than induction of specific cytoskeletal structures (reviewed by Chen 2004).

4

Conclusions and Outlook

In the future combined structural, cell biological, biochemical and gene in-activation approaches will be instrumental in studying the multi-protein assemblies driving cytoskeletal remodelling in cells of the immune system. Determination of the constituents of the involved signalling complexes followed by RNAi-mediated gene silencing and their genetic deletion will help in understanding the exact sequence of events that result in differential T cell responses to diverse stimuli from outside. The identification of new key regulatory components will eventually give rise to new therapeutic drugs for better treatment of disorders involving deregulation of the immune system.

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Membrane-Proximal Signaling Events in Beta-2 Integrin Activation

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Abstract In the immune system, integrins have essential roles in leukocyte trafficking and function. These include immune cell attachment to endothelial and antigen-presenting cells, cytotoxicity, and extravasation into tissues. The integrin leukocyte function-associated antigen-1 (LFA-1), which is exclusively expressed on hematopoietic cells, has been intensely studied since this receptor is important for many functions of the immune system. LFA-1 is involved in a) the interaction between T-cells and antigen presenting cells, b) the adhesion of cells to post-capillary high endothelial venules or to activated endothelium at sites of inflammation (extravasation), c) the control of cell differentiation and proliferation, and d) the regulation of T-cell effector functions. Therefore, a precise understanding of the spatial and temporal control of LFA-1 interaction with its cellular counter-receptors, the intercellular adhesion molecules (ICAM) -1, -2 and -3, in the various contexts, is of high interest. LFA-1 mediated adhesion is induced by several extracellular stimuli in different cell types. In T-cells, LFA-1 becomes activated upon signaling from the T-cell receptor (TCR), and upon cytokine and chemokine sensing. Adhesion of monocytes to ICAM-1 is induced by lipopolysaccharide (LPS), a component of the bacterial cell wall. To investigate the regulation of LFA-1 adhesiveness, research has focused on the identification of interaction partners of the intracellular portions of the integrin alpha and beta subunits. This review will highlight recent developments on transmembrane and intracellular signaling proteins, which have been implicated in beta-2 integrin activation.

Keywords ADAP · Affinity regulation · Beta-2 integrins · CD18 · CD11a · Cytohesin-1 · DNAM · Inside-out · LFA-1 · Rap1 · SKAP-55

1

Introduction

Integrins are heterodimeric plasma membrane receptors, each composed of an alpha and a beta subunit, respectively. These proteins mediate important cell–cell contacts as well as adhesion to the extracellular matrix. They represent a large and diverse family of proteins: 18 alpha subunits and 8 beta subunits have been identified to date which range in molecular weights between

120–170 kDa and 90–100 kDa, respectively. However, due to tissue selective expression and structurally restricted pairing there are probably less than 30 different functional integrin cell surface heterodimers expressed throughout most mammalian cell types. Since cell–cell interactions and cell communication through extracellular components of tissues are so fundamentally important for many biological processes, integrins have been implicated in many functional contexts, the most important of which are maintenance of tissue integrity, leukocyte biology, organ development, wound healing (Hynes 1992) and neurological memory (Grotewiel et al. 1998).

The adhesive properties of integrins are regulated through intracellular signals (inside-out signaling), which in turn are triggered by various extracellular stimuli. The process of integrin activation includes the regulation of ligand binding affinity and/or avidity. Affinity for ligands is modulated through conformational changes of integrin extracellular domains which are induced and controlled by intracellular events. Enhanced avidity for ligands is achieved by clustering of these receptors at the cell surface, a process that might require temporal dissociation of integrins from cytoskeletal restraints allowing lateral mobility of the receptors within the plasma membrane environment.

In the immune system, integrins have essential roles in leukocyte trafficking and function. These include immune cell attachment to endothelial and antigen-presenting cells, cytotoxicity, and extravasation into tissues. The integrin LFA-1 (leukocyte function-associated antigen-1 (LFA-1), which is exclusively expressed on hematopoietic cells, has been intensely studied since this receptor is important for many functions of the immune system (reviewed in: Kinashi 2005). LFA-1 is involved in a) the interaction between T-cells and antigen-presenting cells, b) the adhesion of cells to post-capillary high endothelial venules or to activated endothelium at sites of inflammation (extravasation), c) the control of cell differentiation and proliferation, and d) the regulation of T-cell effector functions. Therefore, a precise understanding of the spatial and temporal control of LFA-1 interaction with its cellular counter-receptors, the intercellular adhesion molecules (ICAM) -1, -2 and -3, in the various contexts, is of high interest.

LFA-1 mediated adhesion is induced by several extracellular stimuli in different cell types. In T-cells, LFA-1 becomes activated upon signaling from the T-cell receptor (TCR), and upon cytokine and chemokine sensing. Adhesion of monocytes to ICAM-1 is induced by lipopolysaccharide (LPS), a component of the bacterial cell wall. To investigate the regulation of LFA-1 adhesiveness, research has focused on the identification of interaction partners of the intracellular portions of the integrin alpha and beta subunits.

This review will highlight recent developments on transmembrane and intracellular signaling proteins, which have been implicated in beta-2 integrin activation.

2

The Integrin Cytoskeletal Anchor Talin

Talin is a 60 nm elongated, flexible, actin-bundling protein consisting of a ~ 50 kDa globular head domain and a ~ 220 kDa rod domain. Two talin molecules form an anti-parallel homodimer. The talin head domain contains a FERM domain (band four point one – ezrin – radixin – moesin) which binds to the NPxY/F motif within the cytoplasmic tails of the integrin beta chains. Through its ability to bind multiple proteins, such as integrin beta chain cytoplasmic tails, F-actin, or the actin binding protein vinculin, talin apparently links integrins to the actin cytoskeleton. On one hand, this may aid in “freezing” integrins in a relatively immobile state. On the other hand, talin enhances the affinity of the integrins for extracellular ligands, probably by spatial separation of the cytoplasmic tails of the alpha and beta subunits, as demonstrated by intramolecular fluorescence resonance electron transfer (FRET) (Kim et al. 2003). Talin mediated separation of the cytoplasmic portions of the alpha and beta subunits likely results in a conformational extension of the extracellular domains. This phenomenon has first been discovered through mutational analysis of the integrin alpha and beta cytoplasmic tails. An arginine in the conserved, membrane proximal GFFKR motif in integrin alpha chains forms a charge pair with an aspartate in the beta-subunit, that interaction being referred to as the “hinge”-domain. Mutants, in which the membrane proximal GFFKR motif of the integrin alpha chain is altered, assume a high affinity conformation (O’Toole et al. 1991). Similarly, expression of a recombinant talin head domain enhances integrin activation (Calderwood et al. 1999). Upon Ca^{++} signalling, the Ca^{++} -dependent protease calpain becomes activated and cleaves talin between the head and rod domain. Subsequently, the bound integrin is released from the actin cytoskeleton which enhances the lateral mobility of the integrin within the plasma membrane and facilitates integrin clustering.

Consequently, siRNA-mediated knock-down of talin leads to a decreased activation of integrins (Tadokoro et al. 2003) and to a reduced adhesion of human peripheral blood lymphocytes (PBL) to ICAM-1 (Shamri et al. 2005). Therefore it has been proposed that talin is required for firm adhesion, and reinforces the cytoskeletal connection that occurs after ligand binding and after clustering of the receptors. Thus, talin appears to have multiple and, possibly, sequential roles in the control of integrin activation.

3

Rap1 and RAPL Control Cell Adhesion

Rap1 is a small GTPase of the ras family which is crucially involved in integrin activation (Bos 2005; Kinashi 2005). The cycling of Rap1 between the inactive

GDP-bound and the active GTP-bound conformation is regulated through several unique guanine-nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Various extracellular stimuli such as TCR signaling, chemokines, LPS, PECAM (CD31) or CD98 engagement lead to an activation of Rap1 (de Bruyn et al. 2002; Reedquist et al. 2000; Bos 2005). Overexpressed Rap1 – or the constitutively activated form, Rap1V12 – potently stimulate adhesion to ICAM-1 upon TCR or chemokine stimulation. Similarly, T-cells from transgenic mice expressing activated Rap1V12 show an enhanced responsiveness to antigenic stimuli and increased clustering of beta1 and beta2 integrins. In contrast, overexpression of a dominant negative mutant of Rap1, Rap1N17, or of a Rap1 GAP (e.g. SPA-1) decreases TCR induced LFA-1 activation and antigen-induced IL-2 production (Katagiri et al. 2000; Sebzda et al. 2002).

The Rap1 responsive element within the integrin LFA-1 cytoplasmic domain has been identified by mutational analysis. The residues KK1097/1099 within the alphaL cytoplasmic domain, adjacent to the conserved GFFKR motif, are critical for Rap1V12 but not PMA induced adhesion (Tohyama et al. 2003). Indeed, a KK/AA mutant for LFA-1 suppresses Rap1V12-induced conformational changes and ligand binding affinity, and impairs binding to ICAM-1 induced by TCR signaling or chemokine stimulation (Tohyama et al. 2003).

In a two-hybrid screen employing activated Rap1V12 as bait, the Rap1 effector RapL was identified (Katagiri et al. 2003). RapL is mainly expressed in lymphoid tissues and contains an RA (ras-association) domain/RBD (ras/Rap1 binding domain) and a C-terminal coiled-coil region. Overexpression of RapL has similar effects to those observed with Rap1V12 in that it promotes adhesion to ICAM-1 and polarized clustering of LFA-1 at the leading edge of antigen or chemokine stimulated T-cells (Katagiri et al. 2004). In the presence of Rap1V12, RapL co-immunoprecipitates with LFA-1, but not with the LFA-1 mutant KK1097/1099AA. However, a direct interaction of RapL with alphaL has not been demonstrated.

In response to chemokine stimulation or to T-cell/APC interaction, RapL rapidly translocates to the leading edge of the polarized T-cell, and a similar relocalization is observed for LFA-1, but not the KK/AA mutant of LFA-1. Therefore, Rap1 and its effector RapL appear to be crucially involved in LFA-1 activation, as well as in determining its subcellular localization. In RapL knock-out mice, lymphocytes and dendritic cells display a severe failure to translocate to their target organs such as lymph nodes and spleen (Katagiri et al. 2004), suggesting that RapL is also required to control migration and homing of immunocompetent cells.

Another Rap1-GTP binding protein of interest is the Ena/VASP and profilin binding protein RIAM. RIAM contains an RA-like domain, a pleckstrin homology (PH) domain and various proline rich motifs (Inagaki et al. 2003). Overexpression of RIAM induces integrin activation and cell adhe-

sion as well as cell spreading and lamellipodia formation, whereas knock-down of RIAM abrogates Rap1 induced adhesion. Since RIAM^{low} cells also show a reduced content of polymerized actin, it appears that RIAM is involved in both the regulation of actin dynamics and Rap1 mediated integrin activation. However, the exact biological role of RIAM needs to be defined.

4

DNAM-1

DNAM-1 (DNAX accessory molecule 1, CD226, PTA-1) is a type 1 transmembrane glycoprotein of the Ig-superfamily. The molecule is expressed on the majority of T-cells and NK cells. PMA stimulation or CD3 engagement in resting T-cells leads to a physical association of DNAM-1 with LFA-1, which is induced by a PKC dependent phosphorylation of Serine 29 within the cytoplasmic domain of DNAM-1 (Shibuya et al. 1999). Upon LFA-1 cross-linking in of anti-CD3 activated T-cells, the src family protein tyrosine kinase fyn becomes activated and phosphorylates Tyrosine 322 in the cytoplasmic domain of DNAM-1. Fyn mediated tyrosine phosphorylation of DNAM-1 is essential for LFA-1 to mediate costimulatory signaling and to trigger IL-12 independent differentiation of naive CD4⁺ helper cells into Th1 cells. Moreover, this phosphorylation is required for LFA-1 induced proliferation of naive CD4⁺ and CD8⁺ T-cells in the absence of IL-2 (Shibuya et al. 2003). Thus DNAM-1 appears clearly involved in LFA-1 co-stimulatory downstream signaling.

Recently it was shown that DNAM-1 binds to the FERM domain containing, actin binding protein 4.1G. Moreover, the C-terminal PDZ-binding motif TRV of DNAM binds to the membrane-associated guanylate kinase homolog (MAGUK) human discs large (hDlg). hDlg interacts with the src-family tyrosine kinase Lck and the Shaker Type potassium ion channel Kv1.3, which are both known regulators of T-cell activation and adhesion (Hanada et al. 1997). Moreover, hDlg, associates with the tumor suppressor PTEN, and the kinesin-like motor protein GAKIN (guanylate-kinase associated kinesin), which links the protein to microtubule-dependent transport functions from the cytosol to the plasma membrane. The enzymatically inactive guanylate kinase-like domain of hDlg also binds the Rap1 GAP Spa-1. It has therefore been proposed that the dynamic associations between DNAM-1, and 4.1G and hDlg may provide a structural basis for a regulated molecular adhesive complex that serves to cluster and to transport of LFA-1 or/and its associated molecules (Ralston et al. 2004).

5

SKAP-55 and ADAP/Fyb/SLAP-130

SKAP-55 and ADAP, preferentially expressed in T-lymphocytes and monocytes are two constitutively interacting cytosolic adaptor molecules which are involved in TCR induced integrin activation and clustering. The src-kinase associated phosphoprotein of 55 kDa (SKAP-55) was initially identified as a Fyn SH2 domain binding protein and contains a unique *N*-terminal region, which is followed by a PH and C-terminal SH3 domain, respectively. Overexpression of SKAP-55 leads to an enhanced adhesion of T-cells to fibronectin and ICAM-1 and promotes LFA-1 clustering. TCR as well as LFA-1 ligation induces translocation of SKAP-55 to lipid rafts. SKAP-55 also localizes to the immunological synapse and increases conjugate formation between T-cells and APCs. Moreover, siRNA mediated knock-down of SKAP-55 expression impairs TCR induced LFA-1 clustering and T-cell/APC conjugate formation and, importantly, this defect can not be rescued by expression of the SKAP-55 homolog SKAP-55R/SKAP-HOM.

The adhesion and degranulation-promoting adaptor protein ADAP was first identified as a 130 kDa protein that binds to the SH2 domain of the cytosolic adapter protein SLP-76, and to the SH2-domain of the Src kinase Fyn (Fyb). ADAP is expressed as two isoforms of 120 and 130 kDa, respectively, which differ by an insertion of 46 amino acids. Both isoforms contain binding sites for the SH2 domains of SLP-76 and Fyn, respectively, two putative nuclear localization sequences, an internal SH3 domain, a proline-rich SKAP-55 SH3 domain binding region, a binding site for Ena/VASP homology 1 (EVH1) domains and a C-terminal SH3-like domain.

T-cells from ADAP knock-out mice show decreased adhesion to ICAM-1 and reduced cytokine production and proliferation upon TCR engagement (Peterson et al. 2001; Griffiths et al. 2001). The expression of ADAP also appears required for SKAP-55 protein stability, since SKAP-55 is degraded in Jurkat cells, lacking endogenous ADAP (Huang et al. 2005). Expression of ADAP restored SKAP-55 stability, but these findings need further elucidation, possibly by siRNA-mediated knock-down of ADAP expression in primary T-lymphocytes. Recently, it was shown that ADAP also associates with – and is phosphorylated by – src in an integrin-dependent manner during osteoclastogenesis. Here, ADAP knock-down cells were shown to be retarded in migration (Koga et al. 2005). SiRNA mediated knock-down of SKAP-55 expression impairs TCR-induced LFA-1 clustering and T-cell/APC conjugate formation. This defect can not be rescued by expression of the SKAP-55 homolog SKAP-55R/SKAP-HOM. In summary, both SKAP-55 and ADAP are crucially involved in TCR induced inside-out signaling, but how these proteins exert their function is incompletely understood.

6

Cytohesins, Cytohesin-binding Proteins, Integrins and the Cytoskeleton

The identification of ARF Nucleotide binding site Opener (ARNO), an ARF-GEF (Chardin et al. 1996) revealed a protein with high similarity to a previously identified integrin binding protein, the closely related cytohesin-1 (Kolanus et al. 1996). Cytohesin-1 and ARNO, which is also termed cytohesin-2 (Frank et al. 1998 a, b) have provided potential roles for ARFs in cytoskeletal and adhesion signaling. A direct role in signaling was demonstrated by the identification of GRP1 (General Receptor for Phosphoinositides1, cytohesin-3) that is capable of binding the lipid messenger PtdIns 3,4,5- P_3 (Klarlund et al. 2000).

The cytohesins share a modular structure, and possess a Sec7 homology domain, encoding the ARF-GEF activity, and a C-terminal pleckstrin homology (PH) domain. This arrangement of domains is also present in other ARF-GEF proteins, but not in the much larger and evolutionarily more distant ARF-GEFs GEA1 and BFG ARF-GEFs. It is currently debated, whether the GEF activities of individual cytohesins show any specificity for particular ARF isoforms (reviewed in Jackson et al. 2000 a, b), but most studies suggest that the cytohesins 1–3 act on multiple ARFs, at least in vitro. However, the ARF-GEFs EFA6A and Tic/EFA6B are reported to show a preference for ARF6 (Derrien et al. 2002; Franco et al. 1999). These differences in substrate specificity may reflect the diverse spatial and functional requirements of individual ARF proteins. Indeed, the cytohesins 1–3 have been detected in the cytoplasm, the plasma membrane, the golgi apparatus and on secretory granule membranes, where multiple ARF species may be present, whereas EFA6A and Tic have so far been shown to exclusively associate with the plasma membrane and a recycling endosomal compartment (Derrien 2002; Franco et al. 1999).

The sequences of cytohesins-1 and -2 were identified by their ability to interact with the c-terminal domain of integrin $\beta 2$, thus indicating a possible role in the process of signaling complex assembly with the cytoplasmic tail of $\beta 2$ integrins (Kolanus et al. 1996). This interaction occurs between residues within the integrin cytoplasmic tail and particular parts of the highly conserved Sec7 domain of the cytohesins (Geiger et al. 2000).

The interaction between cytohesin-1 and the $\beta 2$ tail results in an increased avidity of integrin-substrate binding. However, the GEF activity of cytohesin-1 is not required for this, which indicates that protein–protein interaction alone can regulate this cytohesin-dependent inside-out signaling event (Geiger et al. 2000). The presence of the PH domain is, however, crucial for cytohesins to mediate inside-out signaling, and an isolated PH domain acts as a dominant negative inhibitor (Kolanus et al. 1996). This demonstrates that a correct localisation of the protein in response to the production of PIP_3 is critical for the protein–protein interaction to occur (Nagel et al. 1998 a, b).

In contrast to mediating inside out-signalling via the beta-2 chain, a clear requirement for the GEF activity of cytohesin-1 GEF activity has emerged in the subsequent processes of cell spreading and cell motility (Geiger et al. 2000).

ARF6 appears to be a possible downstream effector of cytohesin-1 GEF activity, and is important for the regulation of leukocyte transendothelial migration (Weber et al. 2001). Cytohesin-3/GRP1 is also able to mediate regulation of cell attachment via $\beta 2$ integrins (Korthauer et al. 2000) and ARNO has been shown to regulate endothelial cell motility (Santy & Casanova 2001, 2002) suggesting that this may be a signaling function shared by each of the closely related cytohesins 1–3.

The polybasic regions of cytohesin-1 and 2 contribute to membrane recruitment, but in cytohesins-1 and -2 this region also contains potential protein kinase C phosphorylation sites. Phosphorylation of cytohesin-1 and -2 by PKC is an event which has been demonstrated in cells and in vitro for cytohesin-1 and -2 (Frank et al. 1998b; Dierks et al. 2001). It has been suggested that phosphorylation of c-terminal residues in cytohesin-2 provides an electrostatic switch that blocks its membrane association, thus providing an additional mechanism for regulation of the interaction of cytohesins with cell membranes and/or other subcellular structures. However, phosphorylation of the respective site in cytohesin-1 does not alter in vitro lipid binding, but leads to its association with the cortical actin cytoskeleton and contributes to PMA stimulated cell adhesion (Dierks et al. 2001). Whether these findings represent differences that are important for the regulation and signaling of these cytohesins, remains to be elucidated.

Besides the conserved Sec7 and PH domains, all cytohesins contain coiled-coil (CC) structures which are often implicated in protein–protein interactions. The most recently identified cytohesin-interacting protein is GRSP1 (GRP1 Signaling Partner 1) a FERM (Band 4.1/Ezrin/Radixin/Moesin homology region; also found in FAK and JAK kinases) domain containing protein, that was shown to bind to cytohesin-3 via an internal CC region. Upon co-expression in CHO cells, cytoplasmic GRSP1–cytohesin-3 complexes are recruited to plasma membrane ruffles following insulin stimulation (Klarlund et al. 2000).

The proteins GRASP (GRP1 Associated Scaffold Protein)/Tamalin, and Cytohesin-binding protein CYTIP (Boehm et al. 2003), also known as Cybr/CASP (Cytohesin Binder and Regulator/Cytohesin Associated Scaffold Protein), contain a PDZ domain, as well as a CC region, that mediates binding to their cytohesin partners (Kitano et al. 2002; Mansour et al. 2002; Nevriy et al. 2000; Tang et al. 2002; Boehm et al. 2003). GRASP is specific for cytohesins-2 and 3, whilst CYTIP can bind to CC regions of cytohesins 1–3. GRASP/Tamalin is a retinoic acid responsive gene product and its overexpression leads to increased plasma membrane association of cytohesin-3 (Nevriy

et al. 2000). In contrast, CYTIP is a cytokine responsive gene originally identified in NK cells. Co-expression of CYTIP with cytohesin-1 leads to redistribution from vesicular structures to the cytoplasm and to co-localisation with membrane ruffles following EGF stimulation (Mansour et al. 2002). CYTIP is also reported to enhance cytohesin-1 ARF-GEF activity in vitro (Tang et al. 2002). Overexpression of CYTIP in T-lymphocytes resulted in inhibition of beta-2 integrin mediated adhesion through sequestration of cytohesin-1 from the plasma membrane to the cytosol (Boehm et al. 2003). In the nervous system GRASP/Tamalin forms a complex between group 1 mGlu receptors and cytohesin-2 which is enriched in postsynaptic membrane fractions where it may participate in distribution of these receptors to neurites (Kitano et al. 2002). Munc13-1 is another binding partner of Cytohesin-1 (Wright & McMaster 2002). Both proteins are associated with regions of high membrane turnover in the presynaptic transmitter release zones (Zaal et al. 1999). Roles for cytohesins-1 and -2 in mediating plasma membrane recruitment of ARF6 have previously been reported in distinct neurosecretory events (Caumont et al. 2000).

7

Cytohesins in Downstream Signaling and Gene Activation Events

A first indication for the involvement of cytohesin-1 in gene activation programs in immune- and other cells emerged from with the finding that cytohesin-1 is recruited to the plasma membrane by the Kaposin A protein, and subsequently mediates cell transformation via the MAP kinase pathway. Kaposin A is a latency-associated transforming protein of the HHV8 virus, which has been implicated in the onset of Kaposi Sarcoma (Kliche et al. 2001).

Furthermore, Perez et al. (2003) have shown that LFA-1 can act as co-stimulatory protein in T cell activation, and that it signals via two proteins that are associated with its cytoplasmic domains, namely cytohesin-1 and JAB-1, respectively. Notably, the cytohesin-1 dependent branch of the pathway appears to require MAP kinase signaling.

Finally, GRP1/cytohesin-3 was identified as a protein that is strongly up-regulated during T cell anergy (anti-CD3 stimulation in the absence of co-stimulation) in the murine system (Korthauer et al. 2000). When tested functionally however, GRP1/cytohesin-3 was found to enhance cell adhesion in a similar fashion as was cytohesin-1, so the possibility remains that other cellular functions are controlled by cytohesin-3 in anergic T cells.

Taken together, cytohesin family proteins and multimeric, cytohesin-containing protein complexes have been strongly implicated in various aspects of cell adhesion and migration in immune cells and in non-hematopoietic cells. A novel emerging theme is their apparent importance for gene transcription events.

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Regulation of Immune Cell Entry into the Central Nervous System

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Abstract The central nervous system (CNS) has long been regarded as an immune privileged organ implying that the immune system avoids the CNS to not disturb its homeostasis, which is critical for proper function of neurons. Meanwhile, it is accepted that immune cells do in fact gain access to the CNS and that immune responses can be mounted within this tissue. However, the unique CNS microenvironment strictly controls these immune reactions starting with tightly controlling immune cell entry into the tissue. The endothelial blood-brain barrier (BBB) and the epithelial blood-cerebrospinal fluid (CSF) barrier, which protect the CNS from the constantly changing milieu within the bloodstream, also strictly control immune cell entry into the CNS. Under physiological conditions, immune cell migration into the CNS is kept at a very low level. In contrast, during a variety of pathological conditions of the CNS such as viral or bacterial infections, or during inflammatory diseases such as multiple sclerosis, immunocompetent cells readily traverse the BBB and likely also the choroid plexus and subsequently enter the CNS parenchyma or CSF spaces. This chapter summarizes our current knowledge of immune cell entry across the blood CNS barriers. A large body of the currently available information on immune cell entry into the CNS has been derived from studying experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. Therefore, most of this chapter discussing immune cell entry during CNS pathogenesis refers to observations in the EAE model, allowing for the possibility that other mechanisms of immune cell entry into the CNS might apply under different pathological conditions such as bacterial meningitis or stroke.

1

The Immune Privilege of the CNS Reconsidered

The traditional view of the central nervous system (CNS) as an immunologically privileged site (Barker and Billingham 1977) was based on the findings that allo- and xenogeneic tissue grafts transplanted into the CNS are much less efficiently rejected by the recipient as compared to orthotopic grafts. The lack of classical MHC class II-positive antigen presenting cells such as dendritic cells (DCs) as well as the lack of constitutive MHC class I and II expression on CNS parenchymal cells and also the lack of lymphatic vessels within the CNS further contributed to the concept of an immune privileged tissue, inaccessible to circulating immune cells. In accordance with this concept, it is well established that homeostasis within the CNS is a prerequisite

for proper electrical activity of neurons, which made it appear quite unlikely that the CNS could tolerate routine immune cell patrolling on the search for relevant antigens.

Protection of the neurons from the constantly changing milieu in the periphery is achieved by the presence of highly specialized endothelial cells lining the walls of CNS microvessels that form the blood–brain barrier (BBB) (Engelhardt 2003) and additionally by the existence of the epithelial cells of the choroid plexus, which establish a blood–cerebrospinal fluid (CSF) barrier (Engelhardt et al. 2001). Both barriers inhibit the free diffusion of molecules from the blood into the CNS and were originally thought to also block immune cell entry into the CNS. The view of the immune privilege of the CNS, where no immune cell entry takes place has, however, been in conflict with observations already made by Medawar and colleagues (Medawar 1948), who demonstrated that an allogeneic tissue graft into the CNS, which would be tolerated in a naive host, is readily rejected in a recipient, who was specifically sensitized to the allo-antigens prior to transplantation. Additional observations of immune responses within the CNS during viral infections, neoplasia, and chronic inflammatory diseases such as multiple sclerosis have provided further evidence that immune responses within the CNS are indeed common and that the CNS is far from being an immunologically ignored organ. Nevertheless, molecular mechanisms regulating immune cell entry into the CNS are different from those effective in other organs. This is exemplified by the observations that injections of the pro-inflammatory mediators lipopolysaccharide (LPS) or tumor necrosis factor (TNF)- α elicit a massive leukocyte infiltrate when injected into peripheral tissues but not, when injected into the brain parenchyma (Andersson et al. 1992a, 1992b).

Thus, the CNS is an immune privileged site, however, this privilege should rather be interpreted in such a way that special rules apply to immune cell entry into the CNS and that the CNS tightly regulates this process in order to balance immunoprotection of this tissue site without sacrificing proper neuronal function.

2

Where do Immune Cells Enter the Immune Privileged CNS?

Immune cells recirculate through the body via the bloodstream. Therefore, in order to access the CNS, circulating immune cells most obviously penetrate the endothelial BBB. The BBB is formed by highly specialized endothelial cells, which inhibit transcellular passage of molecules across the barrier by an extremely low pinocytotic activity and restrict the paracellular diffusion of hydrophilic molecules due to an elaborate network of complex tight junctions (TJ) between the endothelial cells, reviewed in (Kniesel and Wolburg 2000). It should be clarified here that the term “blood–brain barrier” was

originally coined to describe the lack of passive diffusion of molecules across the capillary bed within the CNS, reviewed in (Engelhardt 2003). Leukocyte extravasation does, however, strictly speaking, not take place at the level of the capillary BBB. Rather, although leukocytes do interact with endothelial cells within small capillaries, their extravasation into the tissue occurs at the level of post-capillary venules. It has therefore been questioned whether the specialization of the BBB affects leukocyte trafficking into the CNS at all. Some of the unique characteristics of the CNS capillary endothelial cells do, however, extend to the endothelial cells of the post-capillary vascular segment, thereby equally restricting free diffusion of polar molecules across post-capillary venules. Thus, it seems appropriate to extend the use of the term “blood–brain barrier” to this unique CNS microvascular compartment when discussing leukocyte migration into the CNS.

The endothelial BBB has been considered the most obvious site for circulating immune cells to enter the CNS. Recently, some evidence has pointed to the choroid plexus as an alternative entry site for immune cells directly into the cerebrospinal fluid space (Engelhardt et al. 2001; Kivisakk et al. 2005; Ryan et al. 2005) (Fig. 1). The choroid plexus is a structure organized in a villous surface including an extensive capillary network enclosed by a single layer of cuboidal epithelium (Dziegielewska et al. 2001). It extends from the ventricular surface into the lumen of the ventricles. Its major known function is the secretion of cerebrospinal fluid. The choroid plexus capillaries are fenestrated, while tight junctions surround the apical regions of the choroid plexus epithelial cells forming a blood–cerebrospinal fluid (CSF) barrier (Fig. 1).

In addition to the choroid plexus, there are structures located at strategic positions in the midline of the ventricular system also lacking an endothelial BBB, which are collectively referred to as circumventricular organs (CVOs). Due to their neurohemal or neurosecretory functions, i.e., their neurons monitor hormonal stimuli and other substances within the blood or secrete neuroendocrines into the blood, these areas lack a vascular barrier (Leonhardt 1980). Instead, the capillaries within the CVOs are fenestrated allowing free diffusion of proteins and solutes between the blood and the CVOs. Similar to the choroid plexus a complex network of tight junctions connecting specialized ependymal cells (tanycytes) seal off the CNS from the CVOs (Bouchaud and Bosler 1986; Leonhardt 1980) (Fig. 1). An involvement of CVOs in the communication of the immune system with the nervous system has been established. Due to their fenestrated capillaries the CVOs are often referred to as “windows of the brain” and have been suggested as entry points for pro-inflammatory cytokines into the CNS (Elmqvist et al. 1997). Their localization within the walls of the ventricles and the observation of immune cell recruitment into the CVOs during EAE suggest that the CVOs could also function as possible alternative entry sites for immune cells directly into the CSF (Schulz and Engelhardt 2005).

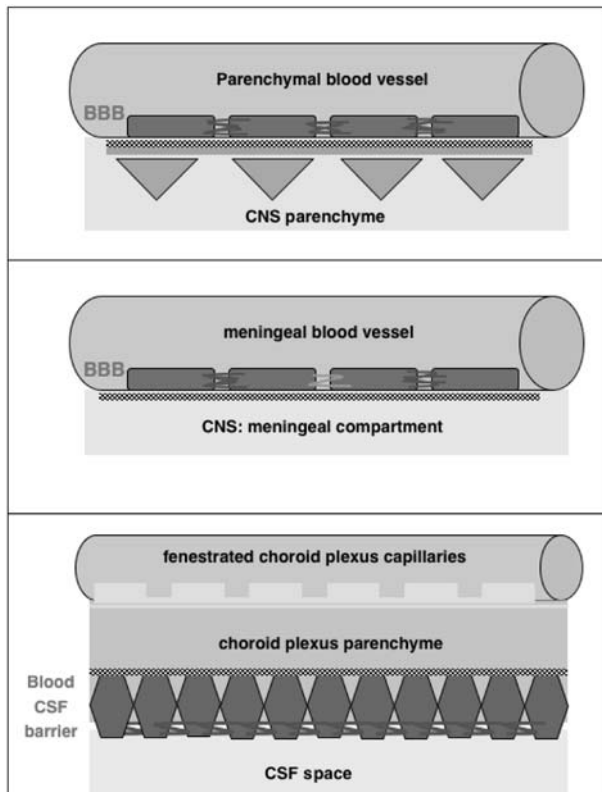


Fig. 1 Schematic presentation of the possible entry sites for immune cells into the CNS. The possible entry sites of immune cells into different CNS compartments and the endothelial and epithelial barriers that have to be crossed by the immune cell are outlined. *Top:* parenchymal endothelial blood–brain barrier with complex tight junctions. *Patterned line* = basal membrane, *grey line* = glia limitans, *triangles* = astrocytic endfeet. *Middle:* meningeal endothelial blood–brain barrier. Note the lack of a glia limitans and astrocytic endfeet. Tight junctions might also be different to those in parenchymal vessels. *Bottom:* To enter the cerebrospinal fluid (CSF) space, leukocytes might leave the blood stream via the fenestrated capillaries of the choroid plexus or the circumventricular organs. To reach the CSF they still have to breach the epithelial blood–CSF barrier built by choroid plexus epithelial cells (hexagonal cells) or tanycytes connected by continuous tight junctions

3

How do Immune Cells Leave the Bloodstream and Enter the Tissue?

It has been well established that immune cells recirculate through the body via the bloodstream and extravasate into the tissue at the level of post-capillary venules. Their successful recruitment across the vascular wall is dependent on the sequential interaction of different adhesion and signal-

ing molecules on the surface of the leukocyte and the respective endothelial cells lining the vessel wall (Butcher 1991; Springer 1990). This multi-step leukocyte recruitment starts with an initial transient contact of the circulating leukocyte with the vascular endothelium, generally mediated by adhesion molecules of the selectin family (L-, E- or P-selectin) and their respective carbohydrate ligands such as P-selectin glycoprotein ligand (PSGL)-1, typically composed of fucosylated and sulfated O-linked sugar residues on different protein backbones. These low-affinity interactions slow down the leukocyte in the bloodstream. Alternatively, at least lymphocytes can use the $\alpha 4$ -integrins $\alpha 4\beta 1$ (VLA-4) or $\alpha 4\beta 7$ to reduce their travelling velocity by interacting with their respective endothelial ligands VCAM-1 or MADCAM-1. After an initial tether, the leukocyte rolls along the vascular wall with greatly reduced velocity and is then exposed to chemotactic factors of the family of chemokines presented on the endothelial surface. Chemokines bind to and activate G-protein coupled receptors (GPCRs) that are expressed on the leukocyte surface (Rot and von Andrian 2004). GPCRs deliver a pertussis toxin-sensitive "inside-out-signal" into the leukocyte thereby activating heterodimeric adhesion molecules of the integrin family (comprising a α - and a β -chain). Integrins are constitutively expressed on the leukocyte surface, however, usually in an inactive conformation (Springer and Wang 2004). Central to integrin function is the dynamic regulation of their adhesiveness through both affinity- and valency-based mechanisms. Upon activation on the leukocyte, integrins change their conformation and cluster thus allowing the firm adhesion of the leukocytes to the vascular endothelium by binding to their endothelial ligands of the immunoglobulin (Ig)-superfamily. Firm adhesion is a prerequisite for leukocyte diapedesis across the endothelium. The molecular events involved in this last step are as yet incompletely characterized. The prevalent view presently held is that transmigration takes place at the endothelial junctions with the leukocyte squeezing through between adjacent endothelial cells. This passage is often envisaged by a zipper-like model, where the traversing leukocyte transiently replaces the homophilic interactions of transmembrane proteins that are localized within the endothelial junctions (Johnson-L  ger and Imhof 2003). However, the current paradigm of junctional migration of leukocytes across the endothelium is challenged by a number of elaborate in vivo and elegant in vitro studies, which provide evidence for a transcellular pathway of leukocyte migration across the endothelium (Carman and Springer 2004; Feng et al. 1998). However, the molecular components of transcellular migration of leukocytes remain unknown.

Thus, as in each step of the multi-step cascade, different receptor-ligand pairs can be used, this process provides numerous possibilities for various combinatorial mechanisms during the recruitment of different leukocyte subsets from the blood to different tissues depending on the expression of distinct traffic signals on the leukocyte surface and on specific traffic signals displayed by the endothelial cells of the microvessels within a given tissue.

Given the specialization of the CNS microvessels, CNS specific traffic signals are expected to be used for immune cell migration across the BBB.

4

The Challenges of Studying Leukocyte Trafficking into the CNS

Investigation of the molecular signals involved in immune cell migration into a given tissue requires both *in vitro* and *in vivo* approaches. When studying immune cell migration across the BBB, both approaches are difficult, and have their pitfalls. Maintenance of the unique characteristics of the BBB endothelium critically depends on the continuous molecular crosstalk with the CNS microenvironment. To date, the identity of the requisite molecules for maintaining BBB characteristics in CNS microvascular endothelium and their exact cellular sources are not known. CNS microvascular endothelial cells, when put into culture, lose many of their BBB specific characteristics, such as high electrical resistance or low permeability to water-soluble tracers (Hamm et al. 2001; Wolburg et al. 1994). Consequently, *in vitro* studies on leukocyte – BBB interactions using primary brain endothelial cells or brain endothelial cell lines still miss certain BBB specific aspects, although these models have proven to be very valuable for the study of leukocyte adhesion and diapedesis across the BBB. In order to maintain BBB specific characteristics in brain endothelial cells in culture, complex *in vitro* BBB models have been established, where brain endothelial cells are co-cultured with astrocytes, pericytes, neuronal cells or even tumor cells (Cecchelli et al. 1999; Rubin et al. 1991). The best BBB characteristics have been achieved with porcine or bovine *in vitro* BBB models, which might explain why these non-rodent models have rarely been used by neuroimmunologists to study leukocyte–BBB interactions. Very recently though, a novel mouse *in vitro* BBB model was described, which opens the doors for future studies on leukocyte–BBB interactions using the large inventory of gene-targeted mouse strains as cellular sources for the dissection of the molecular mechanisms involved (Coisne et al. 2005). Furthermore, reliable analysis of the traffic signals participating in the initial tethering of leukocytes to the BBB *in vitro* requires the application of flow. Although such assays are established (Cinamon and Alon 2003), *in vitro* flow assays specifically addressing leukocyte BBB interactions have not been performed to date.

An alternative possibility to study leukocyte BBB interaction *in situ* is the frozen section “Stamper-Woodruff” assay. This assay has been successfully used by several investigators to establish the involvement of especially $\alpha 4$ -integrins and its endothelial ligand VCAM-1, but also for LFA-1 and ICAM-1 and the lymphoid chemokines CCL19 and CCL21 in lymphocyte binding to inflamed CNS microvessels during EAE (Alt et al. 2002; Steffen et al. 1994; Yednock et al. 1992b).

In vivo homing studies with radioactively or fluorescently labeled cells may be used to track immune cell migration into the CNS. These studies are more difficult because of the fact that, in general, only a low number of immune cells traffic to the CNS (Carrithers et al. 2000). Furthermore, it has been observed that immune cell diapedesis across the BBB takes hours instead of minutes elsewhere (Butcher et al. 1999; Laschinger et al. 2002).

The state-of-the-art approach for studying leukocyte trafficking in vivo is the technique of intravital video microscopy (IVM), which allows for the observation of leukocyte endothelial interactions within the microvessels of a surgically exposed organ in the live anesthetized experimental animal (Mempel et al. 2004; Sumen et al. 2004). In order to observe the CNS microcirculation, three different models of IVM have been established. IVM through the intact skull of very young mice allows for the visualization of superficial brain vessels, probably mostly within the meninges (Piccio et al. 2002). For the study of the CNS microcirculation of adult mice, technically difficult CNS window preparations have to be performed. Surgical removal of a part of the skull preparing a cranial window allows for the observation of both the meningeal microvessels as well as the microcirculation of the cerebral cortex within the CNS grey matter (Kerfoot and Kubes 2002). Additionally, a spinal cord window preparation has been established, which at present is the only model that allows for the viewing of leukocyte–BBB interactions within the spinal cord white matter microcirculation (Vajkoczy et al. 2001).

5

Immunosurveillance of the CNS: Immune Cell Entry into the Healthy CNS

Direct evidence for immune cell entry into the healthy CNS was established by studying experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis, reviewed by (Lassmann 1983; Martin and McFarland 1995; Sospedra and Martin 2005). In contrast to multiple sclerosis, the etiology of which remains unknown to date, EAE is a T cell mediated autoimmune disease of the CNS that can be induced in susceptible animals including different rat and mouse strains by immunization with selected myelin components, reviewed in (Wekerle et al. 1994) or by intravenous injection of freshly activated neuroantigen-specific, i.e., encephalitogenic, CD4⁺ T cell blasts into syngeneic naive recipients (Ben-Nun et al. 1981). In both types of EAE, activation of the CD4⁺ auto-aggressive T cells takes place outside of the CNS. Tracing intravenously injected radioactively labelled encephalitogenic T cell blasts revealed that they are able to penetrate the non-inflamed BBB in healthy Lewis rats (Hickey 1991; Wekerle et al. 1986). It was also established that a high activation state rather than neuroantigen-specificity of the CD4⁺ T lymphoblasts was required for crossing the BBB as resting T cells

irrespective of their antigen-specificity failed to penetrate the BBB in this model (Hickey 1991; Wekerle et al. 1986). In these original studies, radioactively labeled T cells were detected in perivascular locations within the brain parenchyma at the earliest 6 h after their peripheral injection. By observing the migration of the fluorescently labeled encephalitogenic T cell blast into the CNS it was established more recently that 2 h after peripheral injection into mice, T cells can only be observed within the meninges and the choroid plexus parenchyma (Carrithers et al. 2000). Interestingly, the latter study established a role for P-selectin in T cell recruitment into the meningeal and choroid plexus sites. As P-selectin is not stored in the Weibel–Palade bodies of the CNS parenchymal vessels (Mayadas et al. 1993), but minute amounts of constitutive P-selectin are detectable within the CNS (Kerfoot and Kubes 2002), it is tempting to speculate that the restricted expression of P-selectin in endothelial cells of meningeal and choroid plexus microvessels determines different kinetics of T cell entry into selected sites within the CNS. However, intravital epifluorescence video microscopy (IVM) studies of the superficial brain microcirculation failed to detect any interaction of encephalitogenic T cell blasts with healthy CNS microvessels (Piccio et al. 2002). Taken together, these observations suggest that IVM of the brain might fail to detect the rare T cell encounters with CNS microvessels in the healthy animal as observed by the *in vivo* homing studies performed by Carrithers and colleagues.

In contrast, IVM studies of the non-inflamed spinal cord white matter demonstrated the interaction of encephalitogenic T lymphoblasts with the microvasculature at this site, which at the same time was found to be unique (Vajkoczy et al. 2001). T cells were found to interact with spinal cord capillaries and post-capillary venules. Within the post-capillary venules, T cell–BBB interaction was found to be uniquely characterized by a complete lack of T cell rolling and the predominant involvement of $\alpha 4$ -integrin and its endothelial ligand VCAM-1 in both, the initial T cell capture and the subsequent G-protein dependent adhesion strengthening to the spinal cord white matter microvascular wall (Vajkoczy et al. 2001). The integrin LFA-1 (Leukocyte function associated antigen 1, $\alpha L\beta 2$, CD11aCD18) was found to support the migration of T lymphoblasts across the non-inflamed spinal cord white matter microvascular wall and completed diapedesis was first observed 3 h post-injection (Laschinger et al. 2002; Vajkoczy et al. 2001).

These observations suggest that in the healthy CNS, immune cell–endothelial cell encounters within the CNS microvessels are few and that there seem to be differences in the interactions at the vascular wall between meningeal versus parenchymal CNS sites. Molecular and cellular differences for the meningeal and parenchymal microvascular beds have been described (Allt and Lawrenson 1997; Rascher and Wolburg 1997), i.e., meningeal microvessels lack astrocytic ensheathment, conversely, parenchymal microves-

sels lack stored P-selectin (Barkalow et al. 1996; Engelhardt et al. 1997). Furthermore, tight junctions have been described between some endothelial cells of pial vessels, which differ from the complex P-face associated tight junctions found between endothelial cells in parenchymal microvessels, as they leave a discernible gap between adjacent endothelial cell membranes (Allt and Lawrenson 1997). Fenestrated microvessels within the choroid plexus are, strictly speaking, outside of the CNS. As P-selectin was shown to be involved in T cell entry into the meningeal sites and into the choroid plexus during immunosurveillance (Carrithers et al. 2000), it is tempting to speculate that different molecular mechanisms are operating to specifically guide immune cells to distinct CNS compartments. Without antigen-triggered activation, immune cells apparently do not persist behind the BBB and do not properly immigrate across the glia limitans into the CNS parenchyma (Flugel et al. 2001). Thus, immunosurveillance of the CNS is a critical component of host defence and seems without antigen-specific activation of the immune cells behind the BBB to be restricted to the perivascular CNS compartments and the choroid plexus.

6

Immune Cell Migration Across the Inflamed BBB

As already mentioned above, a great body of evidence on the molecular mechanisms involved in immune cell entry into the CNS has been derived from studying EAE. Therefore, most of the observations summarized in the following paragraph stem from the EAE model system.

Once an encephalitogenic T lymphoblast has penetrated the endothelial BBB, it can recognize its specific antigen on antigen presenting cells (APCs) localized within the perivascular space. These APCs were originally described as microglial cells (Hickey 1991) and more recently as dendritic cells (Greter et al. 2005). Antigen-induced activation of auto-aggressive T cells within this compartment is required for initiation and progression of EAE (Greter et al. 2005) including edema formation and inflammation which finally culminate in demyelination. Interestingly, labeled encephalitogenic T cells were found to almost invariably remain within the perivascular area, despite the development of a massive parenchymal inflammatory cell infiltration during ongoing EAE (Cross et al. 1990). Thus, the perivascular space within the CNS seems to serve as a specific “checkpoint” for immune competent cells before the delicate micromilieu of the CNS is put at risk for the benefit of successfully fighting a potential infection.

Antigen-triggered activation of the encephalitogenic T cells behind the BBB initiates CNS inflammation and additional traffic signals will be expressed by BBB endothelial cells allowing the recruitment of additional leukocyte subsets across the BBB.

6.1

Tethering and Rolling Versus Capture

The initial interactions of leukocytes within inflamed CNS microvessels have been studied *in vivo* by performing IVM via a cranial window (Kerfoot and Kubes 2002) or through the intact skull of young mice (Battistini et al. 2003). Tethering and rolling of blood leukocytes was readily observed in inflamed superficial brain and meningeal microvessels during EAE. Rolling was found to be mediated by PSGL-1 and its endothelial ligand P-selectin but additionally by $\alpha 4$ -integrin. Encephalitogenic T cells were observed to roll via PSGL-1 in superficial brain vessel previously stimulated with LPS or TNF- α (Piccio et al. 2002). Appropriate post-translational modification of PSGL-1 was shown to be required for leukocyte rolling in this model (Piccio et al. 2005). Stimulated CNS microvessels were found to express the PSGL-1 ligands E- and P-selectin on their luminal surface as demonstrated by injecting fluorescently labeled anti-E- and anti-P-selectin antibodies. Both, P-selectin and E-selectin were demonstrated to be required for leukocyte rolling in inflamed superficial brain vessels as rolling was reduced after treatment with either anti-E- or anti-P-selectin antibodies and eliminated in E- or P-selectin-deficient mice (Carvalho-Tavares et al. 2000). Interestingly, when human CD4⁺ T cells from MS patients were investigated by IVM through the intact skull of young mice, they were found to roll via $\alpha 4$ -integrin, whereas human CD8⁺ T cells, also derived from MS patients preferentially rolled via P-selectin glycoprotein ligand-1 (PSGL-1) (Battistini et al. 2003). The above described IVM observations, which were to some extent obtained by investigating a xenogeneic system (human T cells in mouse vessels), are in apparent contrast to observations made in the EAE model. EAE studies in the SJL mouse failed to detect expression of E- and P-selectin in parenchymal CNS microvessels and any influence of E- and P-selectin blocking antibodies on the development of EAE (Engelhardt et al. 1997). In accordance with the latter findings, it was shown that although encephalitogenic T cells express the protein PSGL-1, it is only insufficiently decorated with the carbohydrate moieties required for selectin binding (Engelhardt et al. 2005). Consequently, encephalitogenic T cells were shown to poorly bind to P-selectin *in vitro* (Engelhardt et al. 2005). Supporting these observations, antibody inhibition studies in EAE models in wild type SJL mice and EAE studies in PSGL-1 deficient mice have demonstrated that PSGL-1 is not required for immune cell entry into the CNS and the development of EAE (Engelhardt et al. 2005; Osmers et al. 2005). In accordance with the observations of immune cell entry into the healthy CNS, these observations support the conclusion that immune cell migration across inflamed meningeal and parenchymal CNS microvessels appears to rely on different molecular mechanisms. Immune cell recruitment across parenchymal vessels during EAE apparently does not depend on selectins and their ligands, which can obviously be replaced by

other adhesion mechanisms, most probably involving $\alpha 4$ -integrins. In support of this notion is the observation that immune cell entry into the CNS and the development of EAE can be prevented by antibodies that block the $\alpha 4$ -integrins.

6.2

G-Protein Signalling

The requirement for G-protein mediated signalling (and thus an involvement of chemokines) in recruitment of T cell across the BBB during EAE has been demonstrated by several studies (Laschinger et al. 2002; Piccio et al. 2002; Vajkoczy et al. 2001). Many chemokines have been shown to be involved in the pathogenesis of EAE and also have been suggested to be involved in MS pathogenesis. However, these chemokines are mostly produced by astrocytes in the inflamed brain and therefore beyond the BBB (Glabinski and Ransohoff 1999; Ransohoff 2002). In order to prove the role of these chemokines in immune cell recruitment across the BBB, their translocation from within the CNS to the luminal surface of the BBB endothelium needs to be demonstrated (Dzenko et al. 2001). The only chemokines described to be produced by BBB endothelium during EAE are the "lymphoid" chemokines CCL19 and CCL21 (Alt et al. 2002; Columba-Cabezas et al. 2003), which trigger adhesion of encephalitogenic CCR7⁺ T lymphocytes to inflamed brain vessels in Stamper-Woodruff assays (Alt et al. 2002). Thus, although many chemokines are expressed in the CNS in EAE and MS and although several chemokines are definitely involved in EAE pathogenesis, these findings cannot simply be taken as direct evidence for the involvement of these chemokines in leukocyte recruitment across the BBB. The CC chemokine ligand 2 (CCL2)/monocyte chemoattractant protein (MCP)-1, a potent agonist for directed monocyte migration, has been implicated in the pathogenesis of EAE. It has in fact been demonstrated that deficiency in CC chemokine receptor (CCR)2, the receptor for CCL2, confers resistance to EAE induced by immunization with the encephalitogenic peptide of myelin oligodendrocyte glycoprotein (MOG). CCR2-deficient mice failed to develop mononuclear cell inflammatory infiltrates in the CNS and clinical EAE (Izikson et al. 2000). These findings were interpreted such that CCR2 is involved in leukocyte diapedesis across the BBB. However, a second study demonstrated that CCR2-deficient encephalitogenic T cells are able to transfer EAE to wild type mice demonstrating that T cell trafficking across the BBB does not require CCR2 (Fife et al. 2000). Interestingly, CCR2-deficient recipients receiving wild-type T cells failed to develop EAE, suggesting that either CCR2 is necessary for host-derived inflammatory cell – non-T cell – diapedesis across the BBB or that CCR2 is involved in effector functions of inflammatory cells within the CNS (Fife et al. 2000). Although these findings underline the importance of chemokines and their receptors in the pathogenesis of CNS inflammation, the chemokine(s)

mediating the migration of circulating immune cells across the BBB *in vivo* remain to be identified.

6.3

Firm Adhesion

During EAE, the adhesion molecules ICAM-1 (InterCellular Adhesion Molecule-1) and VCAM-1 (Vascular Cell Adhesion Molecule-1) are upregulated on microvascular endothelial cells in the CNS parenchyme (Baron et al. 1993; Cross et al. 1990; Steffen et al. 1994) as well as in superficial brain microvessels (Piccio et al. 2002). The inflammatory cells localized in the perivascular inflammatory cuffs, stain positive for LFA-1 and $\alpha 4\beta 1$ -integrins, the ligands for ICAM-1 and VCAM-1, respectively, but not for L-selectin or $\alpha 4\beta 7$ -integrin (Engelhardt et al. 1998, 2003). Interestingly, in another model of CNS inflammation, where injection of killed *Corynebacterium parvum* induces a mononuclear cell infiltrate in the brain cortex a similar phenotype of inflammatory cells was found with the ability to bind to VCAM-1 but not to MAdCAM-1 *in vitro* (Engelhardt et al. 1995). In MS lesions, ICAM-1 is dramatically upregulated on inflamed vessels (Bo et al. 1996; Sobel et al. 1990), and most infiltrating leukocytes stain positive for LFA-1 (Bo et al. 1996). In contrast, VCAM-1 is not regularly found on endothelial cells in MS lesions (Cannella and Raine 1995), which might be due to VCAM-1 shedding as has been observed on stimulated human brain endothelial cells (Kallmann et al. 2000). The potential functional involvement of these adhesion molecules in leukocyte recruitment across the BBB has been demonstrated in frozen section adhesion assays of EAE brains, where lymphocytes bind via LFA-1 and $\alpha 4$ -integrin to their respective endothelial ligands, ICAM-1 and VCAM-1, on the inflamed cerebral vessels (Steffen et al. 1994; Yednock et al. 1992a). Furthermore, adhesion of encephalitogenic T cells and monocytes to rodent or human brain endothelial cells is mediated via $\alpha 4$ /VCAM-1 and LFA-1/ICAM-1 interactions (Laschinger and Engelhardt 2000; Floris et al. 2002; Greenwood et al. 1995; Seguin et al. 2003).

Blockade of $\alpha 4$ -integrins on leukocytes or VCAM-1 on endothelial cells by using monoclonal antibodies was shown to inhibit or even reverse immune cell infiltration into the CNS and the clinical course of EAE in different animal models (Engelhardt et al. 1998; Yednock et al. 1992a; Baron et al. 1993; Kent et al. 1995; Keszthelyi et al. 1996). These findings supported a central role for $\alpha 4$ -integrins and VCAM-1 in leukocyte-BBB interaction during inflammation. However, discordant effects of anti- $\alpha 4$ treatment were also reported depending on application before or after onset of relapsing EAE (Theien et al. 2003). Clinical MS trials using natalizumab, a humanized anti- $\alpha 4$ integrin antibody produced the most impressive reduction of MS inflammatory disease activity yet reported (Miller et al. 2003) and confirmed the hypothesis of a central role of $\alpha 4$ -integrins in immune cell trafficking into

the CNS. However, shortly post-release, three of several thousand patients that had received natalizumab during the clinical trials developed progressive multifocal leukoencephalopathy (PML), a fatal or catastrophic reactivation of JC virus within CNS glial cells. These dramatic events led to a voluntary suspension of natalizumab for treatment of MS in the beginning of 2005 in order to further investigate the potential risks of anti- $\alpha 4$ -integrin therapy. Irrespective of these devastating findings, it has been learned that during MS leukocyte trafficking into the CNS does rely to a great part on $\alpha 4$ -integrins.

Expression of the $\alpha 4\beta 7$ -integrin ligand MAdCAM-1, was not detected at the BBB during EAE (Steffen et al. 1994, 1996), exempting two situations, namely in EAE in the Biozzi mouse strain (O'Neill et al. 1991) or in EAE transferred by an unusual high number of encephalitogenic T cells (Kanwar et al. 2000). Antibodies blocking $\beta 7$ -integrins ($\alpha 4\beta 7$ and $\alpha E\beta 7$) or specifically blocking the $\alpha 4\beta 7$ -heterodimer do not inhibit the development of EAE in SJL mice (Engelhardt et al. 1998). In contrast, mice deficient for $\beta 7$ -integrins exhibit less severe EAE, when the disease is induced by transferring a very high number of encephalitogenic T lymphoblasts (6×10^8) (Kanwar et al. 2000). Thus, the precise roles of MAdCAM-1 and $\alpha 4\beta 7$ -integrin in EAE pathogenesis remain elusive.

Similarly, the impact of LFA-1/ICAM-1- or LFA-1/ICAM-2 interactions in inflammatory cell recruitment across the BBB during EAE remains to be fully elucidated. Antibody-treatment studies produced contradictory results ranging from reducing to increasing severity of EAE (Cannella et al. 1993; Welsh et al. 1993; Archelos et al. 1993; Willenborg et al. 1993). These apparently discrepant findings most probably result from the use of different reagents, which might additionally alter LFA-1/ICAM-1 interactions of APCs and T cells at the immunological synapse and thus T cell activation (reviewed in (Carlos and Harlan 1994). In contrast, in vitro T cell/brain endothelial cell interaction clearly involve LFA-1/ICAM-1 and to a lesser degree LFA-1/ICAM-2 interactions (Reiss et al. 1998). As encephalitogenic T cells use LFA-1 for their diapedesis across the BBB in the spinal cord white matter in vivo (Laschinger et al. 2002), it remains to be uncovered, how exactly endothelial ICAM-1 and ICAM-2 contribute to immune cell adhesion and diapedesis across the BBB in vivo.

6.4

Diapedesis: Transcellular or Paracellular?

The sequence of molecular steps involved in diapedesis of leukocytes across the BBB are unknown to date. In contrast to the prevalent view that diapedesis of leukocytes takes place through the endothelial junctions, those researchers performing transmission electron microscopy studies on leukocyte diapedesis across the BBB into the CNS during inflammation have invariably

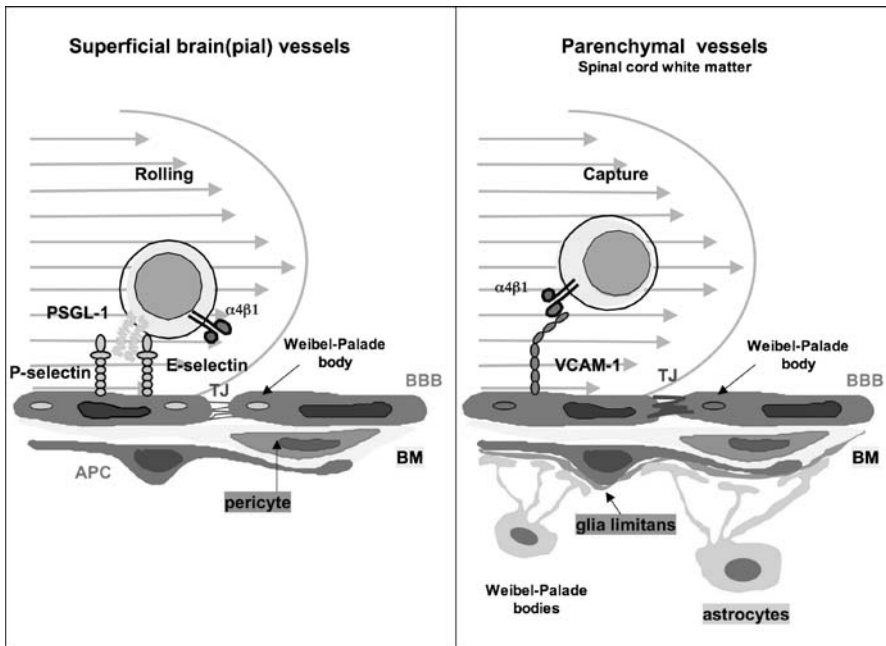


Fig. 2 Initiation of leukocyte BBB interaction within meningeal and parenchymal microvessels in the CNS. In meningeal microvessels endothelial E- and P-selectin and their leukocyte ligand PSGL-1, as well as $\alpha 4$ -integrin are involved in leukocyte tethering and rolling. Meningeal microvascular endothelial cells can express P-selectin and most probably store P-selectin in their Weibel-Palade bodies. In contrast, parenchymal microvascular endothelial cells lack storage of P-selectin in their Weibel-Palade bodies. In deep cortical brain microvessels, which are inaccessible to IVM, the molecular mechanisms of the initiation of lymphocyte recruitment across the BBB might differ, as antibodies directed against $\alpha 4$ -integrin but not against E- and P-selectin or PSGL-1 inhibit inflammatory cell accumulation and EAE. IVM of spinal cord microvessels supports a predominant role of $\alpha 4$ -integrins, which initiate T cell interaction with CNS parenchymal microvessels in the absence of rolling by capture to endothelial VCAM-1. BM = basal membrane, TJ = tight junction, APC = antigen presenting cells

observed it to occur through the endothelial cells, i.e., via a transcellular pathway, leaving the tight junctions intact (Lossinski et al. 1989; Greenwood et al. 1994; McMenamin et al. 1992; Raine et al. 1990; Wekerle et al. 1991; Wolburg et al. 2005). In vitro studies demonstrated that human T lymphocytes can move either through the cytoplasm of human brain endothelial cells or across intercellular contacts between adjacent brain endothelial cells (Wong et al. 1999). In this context, it is interesting to note that during EAE, one study observed neutrophil extravasation across the BBB to occur through the BBB tight junctions, whereas at the same time mononuclear cells probed through the endothelium proper (Cross and Raine 1991). Thus, different leukocyte subpopulations might take different routes of diapedesis across the BBB.

The β 2-integrins LFA-1 and Mac-1 and their endothelial ligand ICAM-1 have been implicated in the diapedesis of leukocytes across endothelium irrespective of the tissue site. Brain endothelial cells lacking ICAM-1 and ICAM-2 no longer support the diapedesis of encephalitogenic T cells *in vitro* (Lyck et al. 2003). The apparent dual role of ICAM-1 in mediating firm adhesion and diapedesis of T cells can be assigned to different parts of the molecule (Greenwood et al. 2003; Lyck et al. 2003). Whereas the extracellular domain of endothelial ICAM-1 suffices to mediate T cell adhesion, the cytoplasmic domain is required to mediate T cell diapedesis probably by inducing RhoA-signalling within the brain endothelial cells (Lyck et al. 2003; Adamson et al. 1999). Activation of RhoA leads to cytoskeletal rearrangements within the endothelium, which are necessary to allow the passage of T cells across the endothelial cell wall (Adamson et al. 1999). As the endothelial actin cytoskeleton is anchored at cell-matrix interaction sites but also at cell-to-cell contacts, involvement of endothelial junctional molecules in leukocyte diapedesis across the BBB is entirely plausible, independent of a paracellular or transcellular route.

Proteins of the family of junctional adhesion molecules (JAMs), localized in endothelial tight junctions have attracted a lot of attention in this context recently, reviewed by (Ebnet et al. 2004; Johnson-L  ger and Imhof 2003; Muller 2003). Antibodies against JAM-A were shown to inhibit neutrophil recruitment in a model of cytokine induced meningitis (Del Maschio et al. 1999) but not in bacterially induced meningitis (Lechner et al. 2000) making its contribution to leukocyte diapedesis across the meningeal microvessels still a controversial issue. Similarly, apparently discrepant observations were made regarding the role of endothelial PECAM-1 in leukocyte trafficking into the CNS. PECAM-1 is localized outside of the structurally defined cell-cell contact zones, i.e., adherens junctions or tight junctions, on endothelial cells also within the BBB. Whereas blocking PECAM-1 was shown to inhibit the accumulation of antigen specific T cells in the CSF after intraventricular antigen infusion (Qing et al. 2001) it was found to be a negative regulator of leukocyte diapedesis across the BBB in EAE. This conclusion was drawn from the observation that PECAM-1-deficient mice suffering from EAE exhibited increased cellular infiltration across the BBB (Graesser et al. 2002). This was accompanied by exaggerated and prolonged cerebrovascular permeability, linking the regulation of BBB permeability and leukocyte diapedesis. These discrepant findings point to a role of PECAM-1 as a context dependent positive or negative signalling molecule rather than a mere adhesion receptor.

Furthermore, CD99, a unique highly O-glycosylated protein that is expressed on many leukocytes and in endothelial cell-to-cell-contacts (Schenkel et al. 2002) has been shown to be involved in the migration of encephalitogenic T lymphocytes across brain endothelial cells *in vitro* (Bixel et al. 2004). Whether CD99 facilitates T cell diapedesis through endothelial junctions via homophilic interactions or rather triggers downstream signalling events ne-

cessary for T cell diapedesis across the brain endothelium independent of a specific route remains to be investigated.

7

Conclusions

It is now well established that immune cells reach the CNS via the bloodstream and can enter the CNS during both health and disease. Traffic signals guiding immune cells across the BBB seem to be different under normal physiological and pathological conditions and might even vary during different stages of inflammation, depending on the inflammatory stimulus and the CNS site involved. Present observations suggest that there are differences in endothelial traffic signals utilized within meningeal *versus* parenchymal or CNS white matter *versus* grey matter, or spinal cord *versus* brain microvessels, which could well result in the recruitment of different leukocyte subpopulations into different sites within the CNS. The immune privilege of the CNS might serve the purpose to regionally regulate the expression of the respective traffic signals to avoid the entry of potentially harmful immune cells such as neutrophils or macrophages into the CNS parenchyma. Continued research will be essential to precisely understand the role of adhesion and signalling molecules involved in the multi-step recruitment of leukocytes across the BBB during health and disease. Once we understand the molecular codes used by the different leukocyte subpopulations (CD4⁺ T cells, CD8⁺ T cells, B cells, monocytes and dendritic cells) to breach the BBB, specific anti-inflammatory therapies might be developed that selectively target the migration of pathogenic leukocyte subpopulation across the BBB, while leaving CNS immunosurveillance untouched. Vice versa, this knowledge might as well be used to direct genetically modified beneficial cells such as killer cells or stem cells across the BBB into the CNS to kill tumors efficiently or to repair damaged tissue.

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Cell–cell communication by Endocannabinoids during Immune Surveillance of the Central Nervous System

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Abstract The immune system is designed to defend the organism from hazardous infection. The way by which cells of the immune system perform this function can be dangerous for the survival and function of the neuronal network in the brain. An attack of immune cells inside the brain includes the potential for severe neuronal damage or cell death and therefore impairment of CNS function. To avoid such undesirable action of the immune system, the CNS harbours an impressive arsenal of cellular and molecular mechanisms enabling strict control of immune reactions – the so-called “immune privilege”. Under inflammatory and pathological conditions, loss of control of the CNS immune system results in the activation of neuronal damage cascades frequently associated with neurological disease. On the other hand, processes of neuroprotection and neurorepair after neuronal damage depend on a steady and tightly controlled immune surveillance. Accordingly, the immune system serves a highly specialized function in the CNS including negative feedback mechanisms that control immune reactions. Recent studies have revealed that endocannabinoids participate in one of the most important ones of the brain’s negative feedback system. The CNS endocannabinoid system consists of cannabinoid receptors, their endogenous ligands and enzymes for the synthesis and degradation of endocannabinoids. It participates crucially in neuronal cell-cell-communication and signal transduction, e.g., by modulating synaptic input and protecting neurons from excitotoxic damage. Over the last decade, it has also become evident that endocannabinoids play an important role in the communication between immune cells, and in the interaction between nerve and immune system during CNS damage. Thus, therapeutic intervention in the CNS endocannabinoid system may help to restore the well-controlled and finely tuned balance of immune reactions in pathological conditions.

1

Keeping Control: CNS Immune Surveillance

The most-prominent element involved in the CNS immune privilege is the blood-brain barrier, an anatomical barrier that consists of a tight endothe-

lial layer, a basement membrane, and a network of astrocyte foot processes, which keeps immune cells out of the brain. The blood-brain barrier is not an absolute barrier: proinflammatory cytokines such as IL-1 can induce expression of adhesion molecules on the endothelial cell surface, facilitate binding of immune cells and induce signals that open the tight junctions, so that immune cells can enter on demand. However, once in the brain, invading immune cells then face a second barrier – the immunological barrier of (1) astrocytes and microglial cells that express death ligands such as CD95 (fasL), which bind to death receptors (CD95, fas) on the surface of invading T cells, (2) astrocytes that express MCH-II, but no co-stimulatory molecules, and therefore induce anergy and apoptosis in T cells and (3) an anti-inflammatory cytokine microenvironment that suppresses invading T cells. Thus, T cell function and survival is strictly controlled and limited in the brain parenchyma and immune reactions are widely regulated and executed by microglial cells, the resident macrophage-like antigen-presenting immunoeffector cell of the brain.

Microglial cells arrive in the CNS before birth have a very slow turnover and express MHC-II- and CD80/CD86 in their activated state. In order to survey the CNS environment, microglial cells express a multitude of different receptors for cytokines, chemokines, prostaglandins and mediators of neuronal injury (Hanisch 2002). In the CNS of a healthy adult, microglial cells are deactivated, display a ramified morphology, and constantly patrol the brain tissue (Nimmerjahn et al. 2005). But to remain deactivated and resting, microglial cells require direct contact with structurally and functionally intact neurons (Hoek et al. 2000) and neuronal electrical activity and neurotransmitter release are constantly recognized by surrounding microglia. In the case of damage, microglia cells become activated in a multi-step-process, which is accompanied by specific morphological, immunophenotypic and functional alterations (Kreutzberg et al. 1996). Intracellular signals of microglial activation include the IL-15-receptor-dependent JAK activity (Hanisch et al. 1997), further serin/threonin-kinases and -phosphatases (Lockhart et al. 1998), the nuclear CREB-protein (Herdegen et al. 1992), NF-kappaB (Lockhart et al. 1998), prostaglandins (Petrova et al. 1999), corticoid-signaling (Drew et al. 2000) and poly-ADP-ribose-polymerase-1 (PARP-1) (Ullrich et al. 2001). After activation, microglia upregulates CNS immune reactions by the release of cytokines, which induce the expression of adhesion molecules on endothelial cells and enhance the permeability of the blood-brain barrier (by TNF-alpha and IL-1), stimulate NK- and TH1-cells (by IL-18 and IL-12) and support B-cell-proliferation and differentiation (by IL-6). Importantly, they produce cytotoxic molecules such as nitrogen and oxygen free radicals, which are capable of damaging neurons directly, but also trigger the release of excitatoric amino acids (Banati et al. 1993). Microglia-derived products are also capable of activating microglial cells themselves, thereby initiating a self-sustaining pathological microglial activation (Takeda et al. 1998). As a result,

sustained pathological over-activation of microglia is considered to be a key event in many acute and chronic neurodegenerative diseases (McGeer et al. 1995, Gebicke-Haerter et al. 1996, Heppner et al. 1998, McGeer et al. 1998, Floyd 1999, Dirnagl et al. 1999). In this situation, inflammatory pathways contribute to the onset and progress neurodegeneration, which provides an interesting opportunity for therapeutic intervention, in particular if the primary pathological event is unknown (e.g., in Alzheimer's) or irreversible (e.g., after stroke).

Here, a crucial question arises: if the immune response is essential for tissue repair in all tissues outside the CNS, does the CNS similarly depend on immune responses for its maintenance and repair, particularly after injury? Since the pioneering work of Michal Schwarz (Molaem et al. 1999), extensive studies have confirmed and described the phenomenon that autoreactive T cells, as well as activated macrophages, are capable of promoting neuroprotection and neuroregeneration in experimental models (Schwartz and Kionis 2005). In fact this is not a very new idea, since in the animal kingdom it is well known that a high degree of immune privilege is associated with a low potential of regeneration. Therefore, it is indeed possible that the inability of the CNS to recover after injury is the price it pays for being an immune-privileged site. Perhaps this is a necessary price to pay in evolutionary terms, in which avoiding risk to healthy individuals is more important than helping injured individuals to survive. The immune privilege could therefore be considered to result from both "tactical necessity" and "calculated risk". Therefore the immune system is allowed to keep the CNS under surveillance, but in a strictly controlled, limited and well-regulated manner.

2

Loosing Control: Inflammatory Escalation during Multiple Sclerosis

During multiple sclerosis (MS), circulating myelin-specific T cells from the lymphatic tissue cross the blood-brain barrier and enter the brain parenchyma, where they engage in close contact with microglial cells, the antigen-presenting immunoeffector cell of the CNS. As a consequence of this contact, microglial cells become activated, stimulate B cells to produce myelin-specific antibodies, and release oxygen and nitrogen free radicals and toxic cytokins, which then attack neurons and the myelin sheath. Together with inflammation, neuronal pathology involving axonal transection plays a pivotal role in MS, and the corresponding animal model disease EAE, even during the early phase of inflammation (Ferguson et al. 1997, Meyer et al. 2001, Trapp et al. 1998, Diestel et al. 2003, Aktas et al. 2005). It has become evident that long-term disability in MS correlates better with axonal damage than with the degree of demyelination (Bjartmar et al. 2003). Moreover, cortical thinning has been demonstrated using MRI techniques

in MS patients (Sailer et al. 2003). Here, a distinct distribution of significant focal thinning of the cerebral cortex in addition to the diffuse cortical atrophy was demonstrated. This focal cortical thinning in the frontal and temporal brain regions was even observed early in the course of the disease, or in patients with mild disability. Patients with longstanding disease or severe disability, however, presented with an additional focal thinning of the motor cortex area. Ongoing axonal degeneration despite blockade of inflammation, and even early widespread axonal pathology without MRI signs of inflammation were interpreted as inflammation-independent axonal damage (Coles et al. 1999, Filippi et al. 2003). We have, however, recently provided the first demonstration of searching T cells coming into direct physical contact with the neuronal cell body, and of related processes in the living brain environment which result in detrimental effects on neurons (Nitsch et al. 2004), whereas unstimulated T cells did not elicit these effects. In chronic neuroinflammation of the CNS, activated myelin-specific T cells are believed to invade the brain and to be reactivated in the parenchyma upon recognition of local autoantigens presented by microglial cells (Flügel et al. 2001). This is supported by the fact that microglial cells have been shown to have the same antigen-presenting capacity as macrophages and dendritic cells (Ulvestad et al. 1994), and can induce anergy or T cell activation according to their state of activation (Matyszak et al. 1999).

Quantitative [(11C)(R)-PK11195 PET, an imaging technique for activated microglia by the detection of "peripheral benzodiazepine binding sites" (PBBS), demonstrated microglia activation not only in areas of focal pathology identified by T(1)- and T(2)-weighted MRI, but also in normal-appearing anatomical structures, including cerebral central grey matter (Banati et al. 2000). The additional binding frequently delineated neuronal projection areas, such as the lateral geniculate bodies in patients with a history of optic neuritis. In a recent study, eight healthy participants and 22 MS patients were investigated via PET, where brain atrophy was significantly greater in MS patients compared to age-matched controls, where [11C]PK11195 uptake increased with the amount of atrophy (Versijpt et al. 2005). Thus, there are strong evidences of an involvement of microglial cells in neuronal damage cascades in EAE as well as in human MS.

Beyond MS, inflammatory processes triggered by microglia are also involved in acute and chronic neurodegeneration such as Alzheimer's disease (Floden and Combs 2005, Giulian et al. 1996, McGeer and McGeer 2001), stroke (Dirnagl et al. 1999, Danton and Dietrich 2003), Huntington's disease (Sapp et al. 2001, Giorgini et al. 2005, Zhang et al. 2005), amyotrophic lateral sclerosis (Turner et al. 2004, Henkel et al. 2004), and also in neuronal damage during CNS infection (Nau and Bruck 2002) and HIV-encephalopathy (Gonzales-Scarano and Garcia 2005). Moreover, activated microglia may actually promote the growth of certain CNS neoplasia (Graeber et al. 2002) and

may detrimental for neurogenesis in the adult brain (Ekdahl et al. 2003, Monje et al. 2003). Thus, in light of the damage properties of activated microglia, it can be assumed that intervention in microglia activation is a therapeutic option for the treatment of neurological diseases in which microglial cells are involved.

3

Maintaining the Balance: The Brain Endocannabinoid System

3.1

Endogenous Ligands

The CNS endocannabinoid system consists of cannabinoid receptors and their endogenous ligands and enzymes for the synthesis and degradation of endocannabinoids (Fig. 1). It is well known to participate crucially in neuronal cell-cell-communication and signal transduction, to control synaptic input, and to protect neurons from excitotoxic damage.

The medical use of *Cannabis* in Europe dates back to the 19th century (O'Shaughnessy 1843, Moreau 1845), when Napoleon's *Commission des Sciences et des Arts* reported the psychotropic, analgesic and antispasmodic effects after the return of its army from Egypt (Rouvier 1810, Christison 1848). Hundred years later, the active compound of *Cannabis* has been identified as the terpenoid derivative Δ^9 -tetrahydrocannabinol (THC) (Adams et al. 1941, Gaoni et al. 1964). Discovery of the active compound then facilitated the development of new potent and selective THC analogues (Melvin et al. 1987) enabled the identification of cannabinoid-sensitive sites in the brain (Devane et al. 1988), which could be identified as the CB1 (Matsuda et al. 1990) and CB2 (Munro et al. 1993) cannabinoid receptor. But surprisingly, the endogenous ligands discovered were lipids, and not peptides as expected. The first endogenous ligand, the first endocannabinoid, the amide of arachidonic acid with ethanolamine, was named anandamide after the Sanskrit word for bliss, *ananda* (Devane et al. 1992).

Metabolic pathways of anandamide (AEA) are unique, and the release of the compound in the living brain is very well regulated (DiMarzo et al. 1994, Giuffrida et al. 1999, Walker et al. 1999). A regulatory key enzyme of anandamide synthesis is the *N*-acyltransferase (NAT), which catalyses the intermolecular transfer of an arachidonic acid group from the sn-1 position of phosphatidylcholine to phosphatidylethanolamine (Fig. 1). Cleavage of the reaction product *N*-arachidonoyl-phosphatidylethanolamine by phospholipase D, which exist in two isoforms in mammals (Kodaki et al. 1997), finally results in the release of anandamide and phosphatidic acid. The activity of NAT activity is Ca^{2+} -dependent and moreover regulated by phosphorylation through PKA. Consequently, anandamide is produced and released by Ca^{2+}

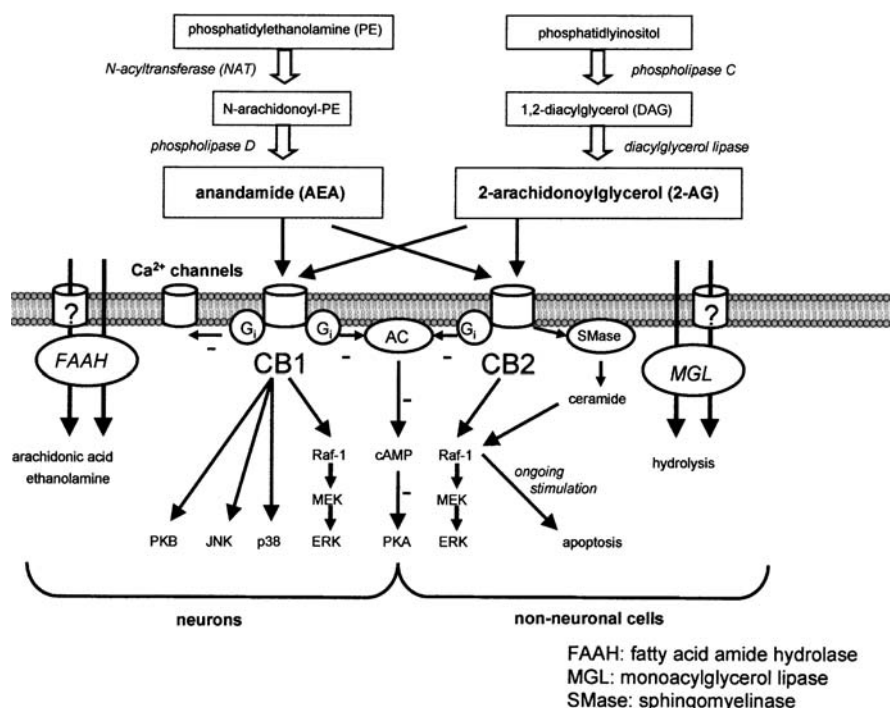


Fig. 1 Schematic diagram of endocannabinoid synthesis, signalling and degradation in neuronal and non-neuronal cells

ionophores and neuronal activity, in particular after stimulation of dopamine D₂-receptors, muscarinic acetylcholine receptors and metabotropic glutamate receptors (Varma et al. 2001, Kim et al. 2002). Dopaminergic neurotransmission stimulates anandamide release by rho-dependent activation of PLD (Senogles 2000) or PLC β -dependent mobilizing of Ca²⁺ (Hernández-López 2000).

Interestingly, the production of anandamide represents an ancient plant signalling system, which is highly conserved in mammalian cells and, therefore, has to be considered as a very successful evolutionary principle. In plants, PLD activity produces fatty acid ethanolamides from long-chain fatty acid phosphatidylethanolamines in response to stress or infection, which regulates the expression of plant immunity-related genes (Chapman 2000).

The second endogenous ligand is 2-arachidonoylglycerol (2-AG): Whereas 2-AG is produced at central intersections of lipid metabolism (Fig. 1) and found in high concentrations in brain tissue, anandamide concentration is about 200-fold lower (Sugiura et al. 1995, Stella et al. 1997) and brain quantities of its precursor *N*-arachidonoyl-phosphatidylethanolamine are probably too little to enable sustained anandamide release. Thus, whereas 2-AG is constantly present in CNS tissue, anandamide is made more on demand. 2-AG

is formed by hydrolyses of 1,2-diacylglycerol (DAG) by DAG lipase (Farooqui et al. 1989) or, alternatively, by synthesis of 2-arachidonoyl-lysophospholipid by phospholipase A1 (PLA1) and subsequent hydrolysis by a lyso-PLC activity (Higgs et al. 1994, Pete et al. 1994). Although similar to anandamide, neuronal 2-AG is produced in response to intracellular Ca^{2+} and stimulation of glutamate receptors, syntheses of anandamide and 2-AG can be independently regulated (Stella et al. 1997, 2001).

Other ligands are noladin ether (Hanus et al. 2001), virodhamine (Porter et al. 2002), and *N*-arachidonoyldopamine (Huang et al. 2002). Noladin ether is an ether-linked analogue of 2-AG that binds to and activates CB₁ receptors (Hanus et al. 2001), while virodhamine is an ester of arachidonic acid and ethanolamine, and endogenous CB₁ antagonist (Piomelli et al. 2003). Their physiological role, however, has yet to be elucidated.

3.2

Release, Uptake and Deactivation

In contrast to small and medium-size peptides, endocannabinoids are highly hydrophobic compounds, and the question how they reach their target structures is not an easy one. One possibility is that endocannabinoids do not leave the cell, but move sideways within the membrane, where they meet their receptors (Xie et al. 1996). This hypothesis has been supported by the identification of an intramembranous binding-site of the CB₁ receptor for anandamide (Song et al. 1996). But endocannabinoids are capable of leaving the cells as soon as they are formed and travel as far as 20 μm from its site of origin (Wilson et al. 2001). Therefore, endocannabinoid binding proteins are suggested (Beuckmann et al. 2000), analogous to serum albumin, which facilitate the transport of anandamids in the blood stream (Bojensen et al. 2003).

Although anandamide and 2-AG can diffuse through lipid membranes, a selective carrier system is suggested that accelerate cellular uptake of endocannabinoids by an energy-independent facilitated diffusion mechanism (Beltramo et al. 1997, Hillard et al. 1997). Anandamide is degraded by fatty acid amide hydrolase (FAAH), an intracellular membrane-bound serine hydrolase (Schmid et al. 1985, Hillard et al. 1995, Ueda et al. 1995, Cravatt et al. 1996). 2-AG is degraded by monoacylglycerol lipase (MGL), a cytosolic serine hydrolase. Both enzymes are widely distributed in the brain. Within the synapse, endocannabinoid-degrading enzymes are expressed asymmetrically: Whereas FAAH is predominantly found postsynaptically, MGL is mostly presynaptically (Dinh et al. 2002). Interestingly, in the hippocampal CA1 field, MGL-positive axon terminals surround cell bodies of pyramidal neurons, which could reflect a functional role of presynaptic MGL in terminating retrograde signalling mediated by 2-AG (Piomelli et al. 2003).

3.3

Signal Transduction

The CB₁ receptor is the most abundant G-protein-coupled receptor in the mammalian brain and is present in the neocortex, hippocampus, basal ganglia, cerebellum and brainstem (Herkenham et al. 1990), where it is primarily found on glutamatergic terminals and axon terminals of GABA interneurons. CB₁ receptors account for most of the behavioural actions of cannabinoid drugs. In its capacity of standard G_{i/o}-coupled receptor (Fig. 1), CB₁ can initiate signalling events typical of this class of transducing proteins – such as closure of Ca²⁺ channels, opening of K⁺ channels, and inhibition of adenylyl cyclase activity – resulting in a decrease in cytosolic cAMP (Howlett et al. 1984). Inhibition of N- and P/Q-type voltage-activated Ca²⁺ channels (Mackie et al. 1992, Caulfield et al. 1992, Twitchell et al. 1997), as well as depression of transmitter release at GABA (gamma-aminobutyric acid) synapses in the CA1 field of the hippocampus (Hoffmann et al. 2000) and at glutamatergic synapses in the dorsal striatum (Gerdemann et al. 2001, Huang et al. 2001) have all been suggested to result from a direct interaction of G_{i/o}-protein beta-gamma subunits with the relevant channels. Presynaptic inhibition at GABA and glutamate synapses also involves cannabinoid regulation of voltage-gated K⁺ currents (Mu et al. 1999). The subsequent regulation of neuronal gene expression by CB₁ depends on the recruitment of complex networks of intracellular protein kinases such as extracellular signal-regulated kinase (ERK) and focal adhesion kinase (FAK), which become activated when hippocampal slices are treated with cannabinoid agonist (Ueda et al. 1995, Cravatt et al. 1996, Derkinderen et al. 1996, 2003). In this context, cannabinoid receptors are coupled to the activation of ERK (Wartmann et al. 1995, Bouaboula et al. 1995a, 1995b, 1996), of c-jun N-terminal kinase and p38 mitogen-activated protein kinase (Liu et al. 2000, Rueda et al. 2000). Moreover, CB₁ receptor is also coupled to the activation of protein kinase B (PKB) (Gomez del Pilar et al. 2000). In summary, cannabinoids inhibit the release of glutamate and other neurotransmitters in neuronal cells by blunting membrane depolarisation and exocytosis (Pertwee 2000, Porter et al. 2001).

Most of the effects of cannabinoids are mediated by their specific receptors CB₁ and CB₂, which are coupled to AC through heterotrimeric G_{i/o}-proteins (Matsuda et al. 1990, Munro et al. 1993). However, in contrast to the CB₁ receptor, transduction systems responsible for CB₂ receptor signalling are less elucidated. CB₂ receptors are expressed mainly by cells of the immune system such as B cells, NK cells, monocytes, neutrophil granulocytes and T cells (Galiegue et al. 1995). In these cells, inhibition of the cAMP/protein kinase A (PKA) pathway may be responsible for the immunosuppressive action of cannabinoids by decreasing the expression of cAMP-responsive genes (Kaminski et al. 1998). CB₂ receptor might control immune-cell proliferation by coupling to ERK activation, a process that is dependent on G_{i/o}-proteins,

but independent of cAMP (Bouaboula et al. 1996). Although CB₁- and CB₂-coupled signalling share some similarities, differences between downstream pathways of both receptors include the ability of CB₁ to modulate Ca²⁺ and K⁺ channels (Pertwee 2000), to phosphorylate ERK independently of protein kinase C (Bouaboula et al. 1996), and to activate PKB (Gomez del Pulgar et al. 2000).

Recently, it has been discovered that endocannabinoids modulate sphingolipid-metabolising pathways by inducing sphingomyelin (SM) breakdown through neutral SMase activation (Kaminski 1998), and by acutely increasing the levels of the second messenger ceramide (Sanchez et al. 1998, 2001) independently of G-proteins. The resulting peak of ceramide accumulation plays an important role in the induction of apoptosis (Galve-Roperh et al. 2000, Guzman et al. 2001). Cannabinoid-induced apoptosis has been investigated in glioma in vitro (Sanchez et al. 1998, Jacobson et al. 2000) and in vivo (Sanchez et al. 2001, Galve-Roperh et al. 2000), where it seems to depend on the sustained generation of ceramide and raf-1-mediated activation of the ERK cascade (Galve-Roperh et al. 2000). In this context, the relationship between activation of the ERK cascade and cell fate has been thought to depend on the duration of the stimulus. Apoptotic action of cannabinoids apparently relies on the long-term peak of ceramide generation and ERK activation (Galve-Roperh et al. 2000), which presumably results from ceramide de novo synthesis rather than SM hydrolysis (Guzman et al. 2001). In PC-12 pheochromocytoma cells, AEA induces superoxide generation, which triggers downstream signals culminating in caspase-3 activation, and finally resulting in apoptosis (Sarker et al. 2000). In addition, VR₁ vanilloid receptor activation by AEA has been reported to trigger apoptosis through a cascade of events (including: cytosolic Ca²⁺ concentration; cyclo- and lipoxygenase activation; a drop in mitochondrial membrane potential; cytochrome c release; and caspase activation) that finally execute apoptotic cell death (Maccarrone et al. 2000). However, the possibility that AEA binds to VR₁ capsaicin receptors (Zygmunt et al. 1999) is conflictive (Szolcsanyi et al. 2000, Piomelli 2001).

3.4

Cell-Cell-Communication

In cells of the immune system, CB₂-receptor-stimulation results in inhibitory signals (Walter et al. 2004) mediated by inhibition of the cAMP/PKA pathway and of the transcription factors NF-AT and AP-1. Endocannabinoids inhibit the CB₂-mediated release of TNF- α , IL-1 β (Klegeris et al. 2003), IL-6 and IL-8 (Berdyshev et al. 1997, Gallily et al. 2000) by monocytes/macrophages, and apparently stimulate nitric oxide release (Stefano et al. 1996). In T cells, THC induces a shift from a Th1- to Th2-type cytokine pattern, such as suppression of IFN- γ and IL-12, and induction of IL-4 release. It has been suggested that endocannabinoids use chemotaxis to attract macrophages and microglial

cells to the site of tissue damage (Walter et al. 2003), where they help to control and limit the local immune response, thus preventing harmful over-activation. Moreover, endocannabinoids are formed by circulating leukocytes and platelets, and induce vascular relaxation by interacting with cannabinoid receptors on the surface of neighbouring endothelial and smooth muscle cells (Batkai et al. 2001). Thus, outside the brain, endocannabinoids are produced on demand, and act on cells located near their site of synthesis (Piomelli 2003).

In the CNS, similar paracrine actions are thought to occur; endocannabinoids mediate a localized signalling mechanism through which principal neurons modify the strength of incoming synaptic inputs (Piomelli 2003). Here, CB₁ receptors are primarily found on axon terminals of GABA-ergic neurons in the neocortex, hippocampal formation, amygdala, basal ganglia, cerebellum, thalamus, hypothalamus, midbrain, medulla, spinal cord and peripheral sensory neurons (Katona et al. 1999, Marsicano et al. 1999, Tsou et al. 1998, Katona et al. 2001, McDonald et al. 2001, Gerdeman et al. 2001, Huang et al. 2001, Hohmann et al. 1999, 2000, Herkenham et al. 1991).

In the CA1 field of the hippocampus, membrane depolarization opens voltage-activated Ca²⁺ channels in pyramidal neurons, producing an elevation of intracellular Ca²⁺ concentrations, which stimulate the synthesis of 2-AG and of AEA. The endocannabinoids then travel backwards across the synapse to interact with CB₁ receptors on axon terminals of GABA-ergic interneurons, leading to the depolarization-induced suppression of inhibition (DSI). Since DSI could facilitate the induction of long-term potentiation in individual CA1 pyramidal neurons (Carlson et al. 2002), endocannabinoids are supposed to be involved in modulation of memory. This retrograde signalling by endocannabinoid-mediated DSI occurs in the hippocampus, where it modulates memory function, but it has also been found outside the hippocampus at interneuron–principal cell synapses of the cerebellum (Llano et al. 1991, Kreitzer et al. 2001).

In the amygdala, anxiogenic stimuli increases anandamide and 2-AG concentrations (Marsicano et al. 2002), which bind to CB₁-positive interneurons in the basolateral complex that processes emotions (Martin et al. 1999), depress glutamate release from axon terminals originating in the cortex (Azad et al. 2003) and decrease the activity of the pyramidal neurons in the central nucleus of the amygdala (Katona et al. 2001). The function of endocannabinoids in controlling affective states in the amygdala has been demonstrated pharmacologically: FAAH inhibitors exhibit marked anxiolytic-like properties (Kathuria et al. 2003), whereas CB₁ inactivation causes anxiety-like and aggressive responses (Navarro et al. 1997, Martin et al. 2002).

The highest densities of CB₁ receptors in the brain are in the terminal fields of striatal projections in the basal ganglia, where they inhibit GABA release and affect motor activity (Romero et al. 2002). In the basal ganglia, anandamide is released by dopamine D₂-receptor activation (Giuffrida et al.

1999) and act at GABA-ergic interneurons and corticostriatal glutamatergic projections (Gerdeman et al. 2001, Hohmann et al. 2000, Herkenham et al. 1991). Clinically, the major role of endocannabinoid signalling in the basal ganglia could be demonstrated by their therapeutic effect in the treatment of L-DOPA-induced dyskinesias (Sieradzan et al. 2001) and Tourette's syndrome (Muller-Vahl et al. 2003)

Endocannabinoid signalling interacts also with pain transmission at multiple sites: Painful stimuli induce release of anandamide, which in turn binds to CB₁ receptors in the periaqueductal grey (Lichtman et al. 1996), rostral ventromedial medulla (Meng et al. 1998) and spinal trigeminal nucleus (Jennings et al. 2001) and mediate an analgesic effect (Iversen et al. 2002).

Endocannabinoids interact not only with GABA-ergic signal transmission, but also with glutamate-dependent signal transmission. Principal neurons in the hippocampus and cerebellum use endocannabinoids to carry out a signalling process that is analogous to DSI, but opposite in sign – a so-called depolarization-induced suppression of excitation (DSE) (Alger 2002). DSE targets glutamatergic rather than GABA axon terminals, and therefore results in reduced excitatory input to the affected cell. Interestingly, glutamatergic terminals are less sensitive to endocannabinoid activation (Ohno-Shosaku et al. 2002), which would indicate that a switch from DSI to DSE might occur when endocannabinoid concentrations at hippocampal synapses attain a certain threshold value (Piomelli 2003). Moreover, acetylcholine release has also been found to be reduced by cannabinoids both in vitro and in vivo (Schlicker et al. 2001, Gifford et al. 1996, Gessa et al. 1998), as has the release of the biogenic amines noradrenaline and serotonin (Schlicker et al. 2001). Since endocannabinoids are clearly involved in striatal LTD (long-term-depression) and hippocampal I-LTD (LTD at adjacent inhibitory synapses), the overall effects of endocannabinoids on hippocampus-dependent learning will surely be an exciting and central subject of future investigation and discussion (Piomelli et al. 2003).

In pathophysiological conditions, endogenous cannabinoids are released after brain injury (Panikashvili et al. 2001, Hansen et al. 2001, Marsicano et al. 2003, Mechoulam et al. 2003, Carrier et al. 2004) and are believed to attenuate neuronal damage by binding to CB₁ receptors (Panikashvili et al. 2001, Hansen et al. 2001, Marsicano et al. 2003), thus protecting against excitotoxicity (Mechoulam et al. 2003, Carrier et al. 2004). Such excitotoxic brain lesions initially result in primary destruction of brain parenchyma, which attracts macrophages and microglia, the major effector cells within the CNS. These inflammatory cells produce large amounts of toxic cytokines and oxygen radicals, which result subsequently in secondary neuronal damage. Since it is known that the cannabinoid receptors, CB₁ and CB₂, are expressed on microglial cells (Facchinetti et al. 2003, Waksman et al. 1999), and that activated microglia are capable of producing larger amounts of endogenous cannabinoids than neurons (Walter et al. 2003), the function of the endocannabinoid

system in neuro-immune-communication during neuronal damage needs to be elucidated in the future.

4

Gaining Control: Therapeutic Intervention in CNS Inflammation

4.1

Microglia as Therapeutic Target

Very recently, Heppner and colleagues directly targeted microglial activities using CD11b-HSVTK transgenic mice and bone marrow chimeras in which a specific lack of microglial activation was achieved (Heppner et al. 2005). Using this approach, inflammatory CNS lesions were repressed, resulting in a marked reduction of the severity of the animal model of MS. Furthermore, the authors demonstrated that microglial cells are crucial for the development of EAE, presumably mediated by the release of cytokines and chemokines as well as reactive oxygen species, as studied in organotypic hippocampal slice cultures. Future studies should examine whether classical neurodegenerative diseases may also benefit from a specific microglia targeting strategy, and how this approach can be translated into clinical application. There are already several therapeutic agents in clinical use or development, which target microglial activities, among other effects. In particular, some *non-steroidal anti-inflammatory drugs (NSAID)* seem to have beneficial effects in neurodegenerative diseases such as Alzheimer's (McGeer and McGeer 2001). Recently, the nuclear receptor peroxisome proliferator-activated receptor-gamma (PPARGamma) agonist *pioglitazone* resulted in a reduction in number of activated microglia with reduced expression of the proinflammatory enzymes cyclooxygenase 2 (COX2) and inducible nitric oxide synthase (iNOS), and a reduction of amyloid deposits in the hippocampus and cortex of an Alzheimer model (Heneka et al. 2005). *Minocycline*, which is known to have neuroprotective properties, was recently shown to reduce the release of cytotoxins from activated microglia (Kradly et al. 2005). However, anti-inflammatory approaches to blocking microglial activation or microglial effector mechanisms are likely non-specific, indiscriminate strategies. Moreover, they do not simply reduce inflammatory damage on the one hand, but they also run the risk of inhibiting protective immunity on the other hand. Therefore, from the clinical point of view, the identification and therapeutic support of anti-inflammatory negative feedback loops might be the better choice because these are only active after immune activation and therefore harbour a low risk of undesired over-inhibition of immune reactions. This means that the therapeutic support of endogenous negative regulatory feedback-loops might be a highly efficient and clinically safe strategy of neuroprotective intervention. Here, the

endocannabinoid system, a major negative regulatory system in the nervous and immune system of the CNS, may represent one promising new therapeutic target in the treatment of acute and chronic neurodegenerative diseases.

4.2

Cannabinoid System as Therapeutic Target

Because of its possible involvement in immune control and neuroprotection, cannabinoids are discussed intensively for treatment of inflammatory disease (Croxford et al. 2005) and moreover as an interesting option in the treatment of neuroinflammation (Correa et al. 2005). Recently we found that the endocannabinoid system is highly activated in patients with MS and identified the release of AEA by inflamed CNS tissue as a new mechanism of neuro-immune-communication during CNS injury, which controls and limits immune response and protect neurons (Eljaschewitsch et al. 2006). A previous study reported elevated endocannabinoid levels in autoimmune encephalomyelitis (EAE), the experimental model of MS, in association with spasticity due to spinal cord pathology (Baker et al. 2001). In EAE, CB₁ receptor stimulation ameliorated spasticity and tremor (Baker et al. 2000, 2004, Arevalo-Martin et al. 2003) and reduced neuronal damage and axonal loss (Pryce et al. 2003), whereas the CB₁ receptor antagonist Rimonabant® transiently worsens signs (Baker et al. 2000, 2001, 2004). Evidence for the participation of endocannabinoid signalling in human MS came from clinical observations that a previously undiagnosed MS exacerbated by Rimonabant® in a person being treated for obesity (van Oosten et al. 2004).

Treatment of MS with the cannabis extracts (Cannador) lowered the frequency of spasms and improved the mobility in a randomized double-blind crossover study (Vaney et al. 2004). Bladder dysfunction has been improved by THC and Sativex in a small-scale open-label study (Brady et al. 2004), as well as patient perceptions of pain and spasticity (Pertwee et al. 2002, Svendsen et al. 2004). On the other hand, no positive effects on spasticity has been reported in patients treated with oral Marinol or Cannador in a blinded trial (Killestein et al. 2002).

In the largest clinical study about the use of cannabinoids for treating symptoms related to MS (CAMS), where 667 people were orally administered over a 15 week period with capsules of THC (Marinol®), capsules of cannabis extract Cannador® or placebo capsules, a subjective improvement of specific symptoms (pain, spasticity, sleep disturbance, walking time), but no effect on spasticity and tremor has been reported (Zajicek et al. 2003). However, in a one-year follow-up of the CAMS study, overall objective improvements on both spasticity (Ashworth Scale) and general disability indices have been found (Zajicek et al. 2004). The results of the long-term CAMS study are particularly encouraging because they indicate that cannabis not only relieves

symptoms but also is potentially neuroprotective and involved in synaptic plasticity; this should be investigated further.

In conclusion, clinical trials ended with both positive and negative results (Pertwee et al. 2002), which could be the consequence of several problems: First, exogenous cannabinoids might interfere locally with endogenous cannabinoids, since they are only partial agonists on cannabinoid receptors. Second, oral delivery of cannabinoid drugs produces a slow and low increase in plasma THC levels (Huestis et al. 1992, Grotenhermen 2003, Wall et al. 1983, Ohlsson et al. 1980, Timpone et al. 1997), whereas a correlation of optimal plasma THC levels with symptom improvement is missing from many studies. Third, the Ashworth scale to measure spasticity might not be sufficiently sensitive to measure small but clinically beneficial effects (Shakespeare et al. 2003). In conclusion, improvements in trial design and outcome measurements are important in clarifying the situation. To overcome problems in the oral delivery of cannabinoids, the sub-lingual spray Sativex® has been developed improvement, which demonstrated beneficial effects on spasticity in patient-assessed scores (visual analogue scale), but no improvement as measured by the Ashworth scale in a placebo-controlled study in 160 MS patients (Wade et al. 2004).

In conclusion, the endocannabinoid system represents a local messenger system within and between the nervous and immune systems, which is apparently involved in learning and memory, emotions, pain processing, regulation of motor functions, control of immune activation and neuroprotection. Therefore, elucidating the integration of the endocannabinoid system in intra- and intercellular signalling networks and their function during physiology and pathophysiology might open new avenues of therapeutic interventions in the future. A successful therapeutic strategy has to maintain or restore the well-controlled and finely-tuned balance of immune reactions, and to protect neurons from inflammatory and non-inflammatory damage.

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